

**APPLICATIONS OF NMR
TO BIOLOGICAL SYSTEMS:**

**CATION-PROTEIN INTERACTIONS
AND PHASE BEHAVIOR IN A
BILE SALT-MONOGLYCERIDE SYSTEM.**

Marianne Svärd

Lund 1985

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MARIANNE SVÄRD

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Abstract The ion binding properties of two calcium binding proteins were studied. Ion binding to parvalbumin was studied using ^{113}Cd NMR. Clear differences between α- and β-parvalbumins were observed when titrating with mono-, di- and trivalent ions. Calcium binding to bone γ-carboxyglutamic acid protein (BGP) from calf was studied by ^{43}Ca NMR. The results could be fitted to a model with a single Ca^{2+} binding site with an association constant in the range of $5.103\text{--}105\text{ M}^{-1}$. The binding of Cd^{2+} to some bile salts was studied by ^{113}Cd NMR. The ^{113}Cd chemical shifts could be fitted to a model with both 1:1 and 2:1 complexes between Cd^{2+} and taurocholate. The phase diagram of the system sodium taurocholate (NaTC)- monoolein (MO)-water was outlined. The phase diagram shows a large homogeneous isotropic phase, a lamellar liquid crystalline phase and a cubic liquid crystalline phase. Hydration and counterion binding in the lamellar l.c. phase was studied by ^2H and ^{23}Na NMR. Self-diffusion coefficients for NaTC and MO in the micellar phase were determined using the ^1H FT PGSE NMR method. Dilution experiments of micellar samples with QLS were made. By combining NMR, QLS and electron microscopy, the coexistence between small micelles and vesicles was revealed.			
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1. LIST OF PAPERS

This thesis consists of a summary and the following papers. In the text, the articles are referred to by their Roman numerals.

- I **NMR studies on parvalbumin phylogeny and ionic interactions**
A. Cavé, A. Saint-Yves, J. Parello, M. Svärd, E. Thulin and B. Lindman (1982) Mol. Cell. Biochem. 44, 161-172.

- II **Metal ion binding to parvalbumin. A ^{113}Cd NMR study of the binding of different lanthanide ions**
T. Drakenberg, M. Svärd, A. Cavé and J. Parello (1985) Biochem. J. 227, 711-717.

- III **Cation binding to parvalbumin studied by ^{113}Cd and ^{23}Na NMR. Peak assignment of rabbit (pI 5.5) parvalbumin**
M. Svärd and T. Drakenberg. Manuscript.

- IV **Calcium binding to bone γ -carboxyglutamic acid protein from calf studied by ^{43}Ca NMR**
M. Svärd, T. Drakenberg, T. Andersson and P. Fernlund. Eur. J. Biochem., submitted for publication.

- V **Cation binding to bile salts. A ^{113}Cd NMR study**
M. Svärd, T. Drakenberg and B. Lindman. Manuscript.

- VI **Micelles, vesicles and liquid crystals in the sodium taurocholate-monoolein-water system. Phase behavior, NMR self-diffusion and quasi-elastic light scattering studies**
M. Svärd, P. Schurtenberger, K. Fontell and B. Lindman. Manuscript.

2. INTRODUCTION

This work deals with cation binding to proteins (I-IV) and bile salts (V). Furthermore, the phase behavior in a ternary system composed of bile salt, monoglyceride and water has been studied (VI). The main experimental method is Nuclear Magnetic Resonance (NMR) and in chapter 2 a short presentation of the different NMR techniques employed is made. In article (VI) the NMR studies are complemented by the use of the Quasi-elastic light scattering technique.

The discussion of the systems mentioned above has been divided into proteins and amphiphiles. The proteins are discussed in terms of cation binding where both ^{113}Cd and ^{43}Ca NMR have been applied to evaluate these properties. The amphiphilic system is mainly discussed from the point of view of phase behavior. The cation binding to bile salts is also discussed.

2.1. Calcium binding proteins

Many calcium binding proteins have been found to act as important messengers or triggers for phenomena in living systems. Examples of such Ca^{2+} regulated events are for instance blood clotting, muscle contraction, cellular fusion and secretion. Phospholipase A_2 is an example of another function of Ca^{2+} ions, namely that of a structural element of an active site of an enzyme. The function of calcium in biochemical and biological processes has been discussed by Kretsinger (1976, 1977).

The binding of calcium to proteins is often referred to as high-affinity or low-affinity binding when the binding constants are in the range of 10^7 - 10^9 M^{-1} and 10^3 - 10^4 M^{-1} , respectively. For even lower values of the binding constant the binding is often referred to as "nonspecific".

High affinity binding sites for calcium exist for instance in the intracellular proteins calmodulin, troponin C and parvalbumin. The calcium concentration within the cell is much lower ($\sim 10^{-7}$ M) than the extracellular concentration ($\sim 10^{-3}$ M). The extracellular calcium binding proteins exhibit a much lower affinity for calcium. Examples of extracellular proteins are prothrombin and bone γ -carboxyglutamic acid protein. These two proteins contain the unusual amino acid, γ -carboxy glutamic acid (Gla). Prothrombin was the first protein which was shown to contain Gla. The protein binds calcium ions and this is an essential step in the process of blood coagulation (Stenflo et al., 1974; Nelsestuen et al., 1974).

In the work presented in this thesis the cation binding properties of two proteins, parvalbumin and bone γ -carboxy glutamic acid protein (BGP) are studied.

The NMR techniques chosen for the study of the two proteins are different. For parvalbumin, the Ca^{2+} ions are substituted by $^{113}\text{Cd}^{2+}$ ions which have suitable NMR properties. The study of Ca^{2+} binding to BGP is made by direct observation of the NMR signals from the $^{43}\text{Ca}^{2+}$ ions.

2.2. Amphiphilic systems

Molecules that at the same time possess a hydrophilic, i.e. water-attracting, and a hydrophobic, i.e. water-repelling, part are termed amphiphiles. The polar group can be either ionic or nonionic while the nonpolar group is often an alkyl chain. In aqueous solutions, the nonpolar groups try to avoid contact with water. This is accomplished through self-association into different types of aggregates. At low concentrations the amphiphile molecules exist as monomers. Above a certain concentration, the critical micelle concentration (CMC), micelles are formed. At even higher amphiphile concentration, lyotropic liquid crystals are formed. Figure 2.1 shows some schematic representations of different types of aggre-

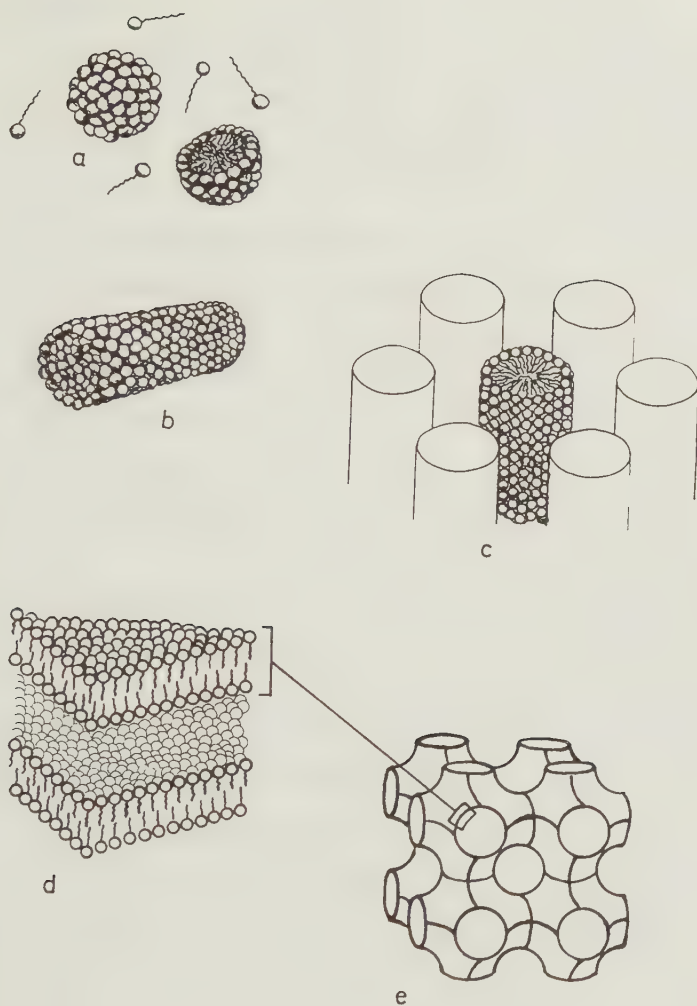


Figure 2.1. Schematic representations of different aggregates and structures that can occur in amphiphilic systems: a) spherical micelle, b) rod micelle, c) hexagonal liquid crystal d) lamellar liquid crystal and e) cubic liquid crystal.

gates or structures that may occur in these systems. For reviews over the amphiphile association behavior see Wennerström and Lindman (1979) and Lindman and Wennerström (1980).

Examples of biological amphiphiles, besides lecithins, are the bile salts and the monoglycerides. .

In combination with lecithin, bile salts act as important solubilizers for mainly cholesterol in the gall-bladder. In the intestine, the bile salts solubilise for instance monoglycerides and fatty acids.

The constituents of gallstones, are mainly cholesterol and calcium precipitates of carbonate, phosphate, bilirubinate or salts of fatty acids. The influence of bile salt on calcium activity has therefore been widely discussed. Some ^{113}Cd chemical shift studies have been performed to investigate the binding of divalent ions to bile salts.

Monoglycerides are formed from hydrolysis of triglycerides in the intestine. One very common monoglyceride found from dietary fat is monoolein.

The bile salts and monoglycerides form together a very interesting system and the ternary system of sodium taurocholate (NaTC)-monoolein (MO)-water has therefore been studied. The phase diagram has been determined and the micellar phase as well as the lamellar liquid crystalline phase have been investigated more thoroughly. The NaTC-MO-Water system represents an important model system also for physico-chemical events in the intestine.

3. METHODS

Almost every element in the periodic table has at least one isotope which may be studied by nuclear magnetic resonance. This fact often offers several different approaches to the study of a problem. The investigation of ion binding to proteins can serve as an illustrative example. The ion binding can either be studied directly from the NMR signal of the interacting ions, or indirectly by observing changes in the NMR spectrum of the protein molecule. A combination of both strategies often gives information of a complementary nature.

3.1. ^{113}Cd NMR in the study of cation binding to proteins and bile salts

^{113}Cd is a spin $I=1/2$ nucleus and the chemical shift of the resonance signal is very sensitive to the type and coordination geometry of the ligands surrounding the ion. The chemical shift range of ^{113}Cd is over 850 ppm (Ellis, 1982; Armitage and Boulanger, 1983).

The Cd^{2+} ion has a radius which is similar to that of the Ca^{2+} ion ($R(\text{Cd}^{2+})=0.97\text{\AA}$ and $R(\text{Ca}^{2+})=0.99\text{\AA}$). Cd^{2+} has in many situations been shown to be a suitable substitute for Ca^{2+} ions. The use of ^{113}Cd NMR in the study of Ca^{2+} -binding proteins has been described by Vogel et al. (1983).

Under slow exchange conditions (i.e. when the metal ion exchange is slow compared to the chemical shift difference between bound and free states) the spectrum shows signals corresponding to the various sites the Cd^{2+} ion can occupy. In the case of parvalbumin, two NMR signals appear which correspond to Cd^{2+} ions bound to the two high affinity sites of the protein.

When fast exchange conditions prevail (the chemical exchange rate of Cd^{2+} ions is fast compared to the chemical shift difference between bound and free states) a single signal appears with a chemical shift which is a population weighted average of the chemical shifts of free and bound ions. An example of such a system is when sodium taurocholate is titrated with $^{113}\text{Cd}^{2+}$ ions.

3.2. Quadrupolar nuclei in isotropic systems: ^{23}Na and ^{43}Ca in the study of ion binding to proteins

^{23}Na and ^{43}Ca have spin quantum numbers greater than 1/2 (3/2 and 7/2, respectively). They thus possess electric quadrupole moments which makes a direct observation of the "bound" ions difficult (Andersson et al., 1982). When intermediate exchange conditions apply (the exchange rate of the metal ions is of the same order as the relaxation rate of the bound ions) the line shape of the observed NMR signal contains information about the dynamics of the binding (Drakenberg et al., 1983). A combination of a temperature- and metal ion dependence of the NMR signal can, under fortunate conditions, give both the exchange rate and binding constants.

The experimental problems when working with low metal ion concentrations limits the applicability of the method. At the present time it is not possible to determine binding constants larger than 10^4 M^{-1} .

3.3. Quadrupolar nuclei in anisotropic systems: ^2H and ^{23}Na NMR in lyotropic liquid crystals

The use of quadrupolar nuclei in the study of liquid crystalline systems has become increasingly important over the last decades. For reviews of the subject see Davis (1983) and Seelig (1977). In an anisotropic system (such as the lamellar and hexagonal l.c. phases) the quadrupolar interaction is not completely averaged to zero. The residual quadrupole interaction is seen as a splitting of the NMR signal. The strength of

the quadrupole interaction can be measured from the peak-to-peak distance (the splitting, Δ) in the spectrum.

The observed splitting is an average due to fast diffusion of the nuclei within the microcrystallites, over all of the different sites and can therefore be described as:

$$\Delta = \left| \sum_i p_i \Delta_i \right| \quad (1)$$

where p_i is the fraction of ions or molecules in site i .

The ions or molecules in the bulk water are experiencing an isotropic environment. The contributions to the observed splitting comes from the ions or molecules at or near the aggregate surface, i.e. the "bound" state (indicated below by the subscript b).

$$\Delta = \left| \sum_i p_{b,i} \Delta_{b,i} \right| \quad (2)$$

In a practical situation of phase diagram studies, the "normal" water of the system is substituted by heavy water. Since diffusion between microcrystallites is slow the ^2H spectra from different phases in the sample will become superimposed. Consequently, the presence of both the lamellar and the hexagonal phases is revealed in the NMR spectrum through two splittings. Information about the hydration of the aggregate surface can also be obtained from water deuteron splittings.

The ionic interactions in lyotropic liquid crystals can be studied from NMR quadrupole splittings such as ^{23}Na . The general principles of this type of study have been described by Wennerström et al. (1974).

3.4. Self-diffusion coefficients from the ^1H FT-PGSE technique

Self-diffusion coefficients for several different molecules in a system can conveniently be obtained through the ^1H Fourier Transformed Pulsed Gradient Spin Echo NMR technique. The method has been developed quite recently by Stilbs (1982, 1983) and Stilbs and Moseley (1980).

In the experiment, the signal intensities are measured as a function of the duration of an applied magnetic field gradient pulse. After a 90° pulse, the magnetic spins start to dephase during a time τ . The magnetic spins refocus after a 180° pulse and at a time 2τ , a spin echo is obtained. If the molecules diffuse the spins will not be completely rephased after a time 2τ . The attenuation of the signal intensities due to diffusion in an applied field gradient follows the expression

$$\frac{A_i}{A_0} \propto \exp \left[-(\gamma G \delta)^2 D (\Delta - \delta/3) \right] \quad (3)$$

where γ is the magnetogyric ratio, G is the strength and δ is the length of the field gradient. D is the diffusion coefficient and Δ is the time between the magnetic field gradient pulses.

4. CALCIUM BINDING PROTEINS: CATION-PROTEIN INTERACTIONS

The activity of many proteins and enzymes are dependent of the presence of Ca^{2+} ions. It has been shown that the binding of Ca^{2+} ions often is connected with a conformation change resulting in an active form of the protein (or enzyme). Variations in the Ca^{2+} concentrations may consequently result in the triggering of physiological events. Examples of such Ca^{2+} regulated functions are the contraction of muscles and the phosphorylation of proteins.

Calcium binding proteins are found both intra- and extracellularly. An important difference between these two environments is that the intracellular Ca^{2+} concentration is much lower than the extracellular concentration (10^{-7} M compared to 10^{-3} M). As a result of this the intracellular Ca^{2+} binding proteins generally exhibit a much higher affinity for Ca^{2+} compared to the extracellular proteins. In the work presented in this thesis two proteins representing these two types are studied, parvalbumin and bone γ -carboxy-glutamic acid protein (BGP).

4.1. Parvalbumin

Parvalbumins form a class of low molecular weight ($M_r \approx 11500$), Ca^{2+} -binding proteins found in rather high concentrations in the sarcoplasm of vertebrate skeletal muscles. The parvalbumins possess acidic isoelectric points (pI) and in the following discussion, the proteins are referred to by their respective pI-values. The physiological function of parvalbumins is not known but it has been suggested that the protein plays an important role in the relaxation mechanism of the muscle (Pechere, 1977).

The proteins in the parvalbumin family all show high affinity for Ca^{2+} ($K_a \approx 10^9 \text{ M}^{-1}$). The crystal structure has been determined for one of the parvalbumins, the component pI 4.25 from carp. (Kretsinger & Nockolds, 1973).

^{113}Cd NMR has been shown to be an ideal probe for the high affinity metal binding sites in calcium binding proteins (Drakenberg et al., 1978; Forsén et al., 1979; Bailey et al., 1980). The Ca^{2+} ions in the native protein can readily be substituted by $^{113}\text{Cd}^{2+}$. For parvalbumin the two $^{113}\text{Cd}^{2+}$ ions give rise to two separate NMR signals corresponding to the EF and CD sites. There are six α -helical segments in parvalbumin, denoted A to F and the binding sites are formed by the loops in between the C-D and E-F α -helices respectively. The binding sites are therefore termed the CD and EF sites (see figure 4.1.). It has been postulated by Kretsinger (1976) that many high-affinity calcium binding proteins possess an "EF-hand" type of binding site. The two calcium binding proteins, calmodulin and troponin C (Tnc) give rise to similar ^{113}Cd NMR spectra when Ca^{2+} is substituted by $^{113}\text{Cd}^{2+}$ (Forsén et al., 1979, 1980).

The parvalbumins have been divided into two phylogenetically different classes, α and β on the basis of their primary structures (Goodman & Pechere, 1977; Goodman et al., 1979).

In **Article I** some α - and β -parvalbumins have been studied. The Ca^{2+} ions were replaced by $^{113}\text{Cd}^{2+}$ which allowed the direct observation of the Cd^{2+} ions bound to the protein using ^{113}Cd NMR. Competition experiments were performed which clearly demonstrate differences in cation binding properties between the α - and β -parvalbumins.

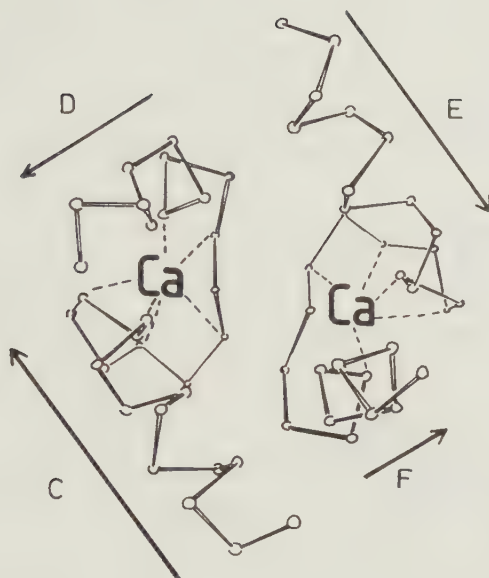


Figure 4.1. Schematic representation of the α -carbons around the Ca^{2+} binding sites in carp. (pI 4.25) parvalbumin (Kretsinger & Nockolds; 1973).

The assignment of the ^{113}Cd signals to the EF and CD sites were in **Article I** based upon a previously made Cd^{2+} - Gd^{3+} competition experiment (Drakenberg et al., 1978). This assignment has, however, in **Article II** been re-evaluated. In Figure 4.2 the ^{113}Cd NMR spectral appearance is shown as Tb^{3+} is added to the $^{113}\text{Cd}^{2+}$ loaded carp parvalbumin. Sowadski et al. (1978) showed that Tb^{3+} selectively replaces Ca^{2+} in the EF-site before replacing Ca^{2+} in the CD-site. It has been observed by Ragg et al. (1985) from ^1H NMR that Ca^{2+} and Cd^{2+} saturated parvalbumin give rise to very similar spectra. Consequently, the

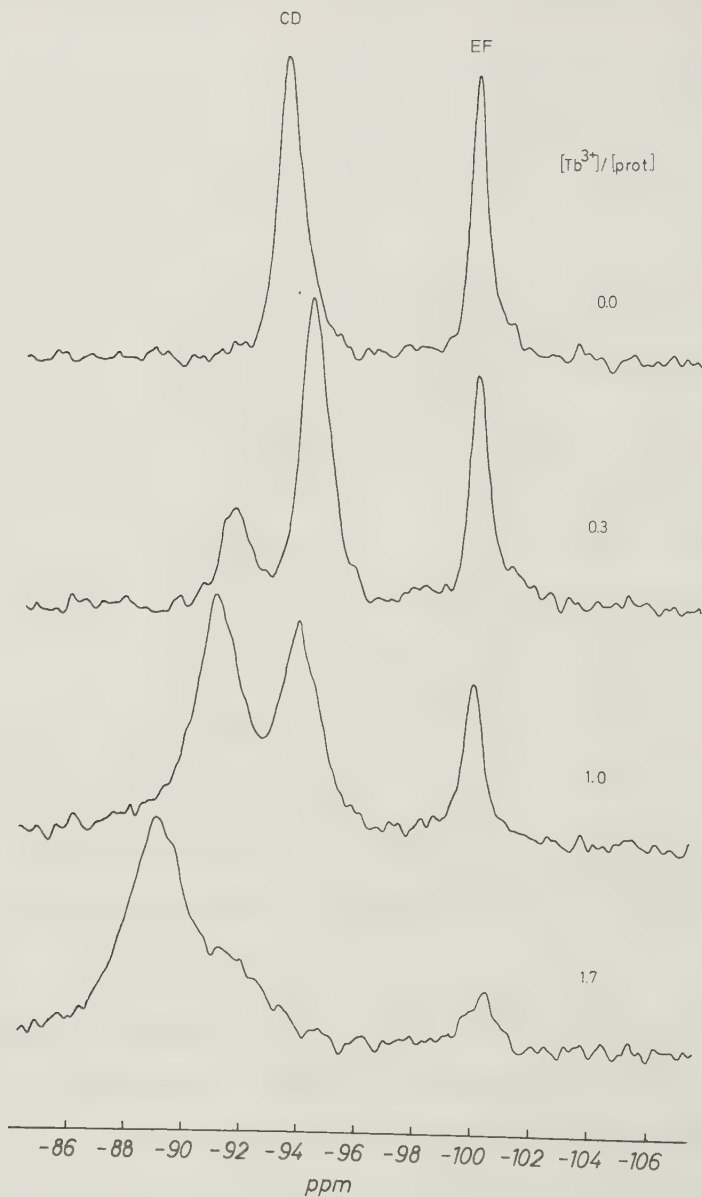


Figure 4.2. ^{113}Cd NMR spectra of Cd^{2+} saturated carp (pI 4.25) parvalbumin as a function of added Tb^{3+} . Indicated in the figure are the Tb^{3+} /parvalbumin ratios.

high-field ^{113}Cd signal in Figure 4.2 can be assigned to Cd^{2+} in the EF site. The other two signals in the spectrum correspond to Cd^{2+} in the CD site when there is either Cd^{2+} or Tb^{3+} in the EF site. In **Article II** it was further concluded that the peak assignment for pike pI 5.0 (α -lineage) is the reverse i.e. the low-field ^{113}Cd signal corresponds to the EF-site.

The chemical shift of one of the ^{113}Cd NMR signals, $\text{CD}(\text{Cd}^{2+})$, of the β -parvalbumins was shown in **Article I** to be sensitive to additions of cations like Mg^{2+} , Na^+ , Ca^{2+} , NH_4 . The α -proteins showed, on the other hand, no such dependence.

The effect of mono- and divalent ions on the chemical shift is attributed to the existence of a third, external, binding site in the β -proteins close to the CD high affinity site. The addition of the paramagnetic Mn^{2+} ion to the Cd^{2+} loaded carp parvalbumin (pI 4.25) also caused line broadening of the signal from the CD bound ^{113}Cd . From this selective broadening effect by Mn^{2+} the distance between the third site and the high-affinity CD site was estimated from the Solomon-Blombergen equation (Dwek, 1973), to be around 5Å.

In **Article II** it is proposed that the α -lineage pike protein pI 5.0 also exhibits a third site which is weaker than the third site described above.

When Mn^{2+} is added to the cadmium loaded α -parvalbumin of pike pI 5.0 both signals are broadened although one of the signals is somewhat more affected and correspond to $^{113}\text{Cd}^{2+}$ bound to the CD site(**II**). Due to this line broadening effect of the Mn^{2+} the same procedure was used in **Article III** for the assignment of the $^{113}\text{Cd}^{2+}$ signals of rabbit pI 5.5 parvalbumin. For rabbit parvalbumin (pI 5.5) the high-field signal correspond to the CD site bound $^{113}\text{Cd}^{2+}$. Thus, the assignments of the

^{113}Cd NMR signals are the same for this protein as for carp (pI 4.25) parvalbumin even though the rabbit (pI 5.5) parvalbumin belongs to the α -lineage while carp pI 4.25 belongs to the β -lineage.

In **Article III** the effect of $^{113}\text{Cd}^{2+}$ added to the apo-parvalbumins of carp (pI 4.25), pike (pI 5.0) and rabbit (pI 5.5) was studied. It was found that Cd^{2+} binds with slightly higher affinity to the CD-site of the pike parvalbumin while for carp and rabbit it is the EF-site that possess slightly higher affinity for Cd^{2+} ions.

It has been shown that the calcium free protein is clearly more flexible and that the binding of calcium induces substantial conformational changes (Nelson et al., 1976). The multitude of ^{113}Cd signals appearing when the apo-parvalbumin is titrated with Cd^{2+} may be due to changes in conformation with the Cd^{2+} concentration.

Sodium binding to parvalbumin has been studied from ^{23}Na NMR by Grandjean et al. (1977) and Parello et al. (1979). Grandjean et al. (1977) found that Na^+ binds to one of the Ca^{2+} sites in a half-decalcified parvalbumin (pike pI 5.0), while Parello et al. (1979) did not find any significant binding to either the native protein or to the apo-parvalbumin (hake pI 4.36). Parello and coworkers were able to demonstrate that the broadening of the ^{23}Na signal observed by Grandjean et al. is due to Na^+ ions binding to an EGTA-parvalbumin complex.

More recent studies show, however, that there is a relatively small broadening of the ^{23}Na NMR resonance in the presence of Ca^{2+} -loaded parvalbumin. This broadening is, however, only observed at low Na^+ concentrations (**III**). The results from a Na^+ - Mg^{2+} competition experiment clearly shows that the two ions compete for the same site. From ^{113}Cd NMR it has been demonstrated that Mg^{2+} does not compete with Cd^{2+} for the high affinity sites (**I**). The conclusion is therefore that the Na^+ binding observed can be attributed to the third site.

4.2. Bone γ -carboxyglutamic acid protein (BGP)

Bone γ -carboxyglutamic acid (BGP), sometimes also called osteocalcin, is a small protein ($M_r \approx 5700$) having a moderately high affinity for Ca^{2+} . Characteristic for the protein is the presence of the amino acid, γ -carboxyglutamic acid (Gla). See figure 4.2.

A review over Gla-containing proteins has been made by Gallop et al. (1980).

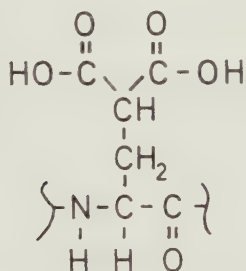


Figure 4.2. γ -carboxyglutamic acid (Gla)

The protein binds strongly to hydroxyapatite and has been suggested to play a regulatory role in mineral deposition and calcium apatite crystal growth (Hauschka and Gallop, 1977). BGP is predominantly found in bone but the protein has also been detected at low levels in the blood. The presence of BGP in the blood may have clinical applications. Elevated levels of BGP in the plasma have been associated with certain types of bone diseases (Price et al., 1980; Gundberg et al., 1983).

The amino acid sequences of BGP from human, monkey, chicken, cow, rat and swordfish have been determined (Hauschka et al., 1982 and references

therein). The positions of Glu-residues at 17, 21 and 24 are invariant and the disulfide bond between Cys-23 and Cys-29 is also present in BGP from all species investigated so far. The binding of Ca^{2+} to BGP is well documented from several studies. Chicken BGP has been reported to bind 4 Ca^{2+} with binding constants in the range of 10^3 - 10^4 M^{-1} (Hauschka and Gallop, 1977). From equilibrium dialysis, bovine BGP was shown to bind 1 to 2 Ca^{2+} ions with a binding constant of $3 \cdot 10^3 \text{ M}^{-1}$ (Gundlach and Voegeli, 1983).

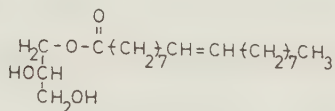
In **Article IV** ^{43}Ca NMR has been used to study Ca^{2+} binding to BGP from calf. The ^{43}Ca NMR linewidth was studied as a function of Ca^{2+} concentration, temperature and pH. From an iterative calculation procedure (Drakenberg et al., 1983) it was concluded that there is at least one site with K_a in the range of $5 \cdot 10^3$ - 10^5 M^{-1} . From the temperature dependence of the ^{43}Ca NMR signal the metal ion exchange was calculated to be $k_{\text{off}} = 4 \pm 0.5 \cdot 10^3 \text{ s}^{-1}$.

The pH dependence of the metal ion NMR signal shows that the binding of Ca^{2+} is sensitive to an apparent $\text{p}K_a$ value of 5.1.

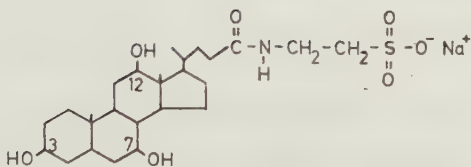
The amount of protein needed for the NMR experiments is about 100 mg. In **Article IV** there is also included a large scale purification procedure for calf BGP. The method of using EDTA to dissolve the pulverized bone material is often employed. However, in order to avoid any interference from a strong chelator like EDTA in the study of metal ion binding, the bone was instead dissolved in 30% acetic acid. The acetic acid dissolving procedure is not, however, as efficient as the EDTA procedure.

5. BIOLOGICAL AMPHIPHILIC SYSTEMS

In the work presented here two biological amphiphiles, sodium taurocholate (NaTC) and monoolein (MO) are studied. Their structural formulae are shown in Figure 5.1.



1-MONOOLEIN



SODIUM TAUROCHOLATE



Figure 5.1. Structural formulae of 1-monoolein (MO) and sodium taurocholate (NaTC). A space filling model of NaTC is also shown.

A monoglyceride such as monoolein is a nonionic "swelling" amphiphile with very low water solubility. At very low concentrations, monoolein separates as a cubic liquid crystalline phase and at high monoolein concentration there is a lamellar liquid crystalline phase. Sodium taurocholate is built up from a steroid skeleton. Three hydroxyl groups at positions 3, 7 and 12 and a sulfonate group render the molecule its hydrophilic character.

Bile salts are synthesized in the liver from cholesterol and form mixed micelles together with lecithin in the gallbladder or are secreted into duodenum where they act as a solubilizer for lipophilic water insoluble material.

The most common bile salts are summarized in Table 5.1.

Table 5.1. The most abundant bile salts in man. They are conjugated with taurine^a or glycine^b at C-24 of the steroid skeleton

BILE SALT	
Trivial name	Systematic name
cholate	3 α , 7 α , 12 α -trihydroxy-5 β -cholanoate
chenodeoxycholate	3 α , 7 α -dihydroxy-5 β -cholanoate
deoxycholate	3 α , 12 α -dihydroxy-5 β -cholanoate
ursodeoxycholate	3 α , 7 β -dihydroxy-5 β -cholanoate
lithocholate	3 α -monohydroxy-5 β -cholanoate

a) $\text{H}_2\text{NCH}_2\text{CH}_2\text{SO}_3\text{H}$

b) $\text{H}_2\text{NCH}_2\text{COOH}$

In the body the bile salts are conjugated with either taurine or glycine. (The ionic group is a sulfonate or carboxylate, respectively). The pK_a value for glycine conjugated bile salts are between 4 and 5 while for taurine conjugated bile salts it is around 2. All of the taurine conjugates and most of the glycine conjugates are therefore ionized at physiological pH.

The bile salts are not known to form any liquid crystalline phases. Deoxycholate forms, however, a gel type of structure under special conditions (Blow and Rich, 1960). It has also been observed that calcium cholate forms a type of gel at very low concentrations (M. Svård, unpublished results). The trihydroxy bile salts form very small micelles with an hydrodynamic radius around 10Å (Mazer et al., 1979). Furthermore, these "atypical" amphiphilic molecules lack the distinct CMC-value which is found for the more conventional long chained surfactants (Kratohvil et al., 1983; Mukerjee et al., 1984).

5.1. Cation binding to bile salts

Bile salts form, as mentioned above, mixed micelles with lecithin which can solubilize cholesterol. The effect of bile salts on the Ca^{2+} activity in the gall-bladder has been extensively discussed (see for instance Moore, 1984). The reason for this is that, besides cholesterol, Ca^{2+} -salts of fatty acids, calcium carbonate and calcium phosphate are found in gall-stones. The therapeutic use of certain bile salts (ursodeoxy- and chenodeoxycholic acids) to patients with small gall-stones is an accepted treatment. The objective for such a treatment is to dissolve the gall-stones and in this connection it is thus important to consider Ca^{2+} binding and not only cholesterol solubilization.

The ^{113}Cd NMR chemical shift is sensitive to the number and type of ligands surrounding the Cd^{2+} ion. **Article V** describes a ^{113}Cd NMR chemical shift study of cation binding to some bile salts.

The bile salt chosen was mainly the Na^+ salt of taurocholate for which the ^{113}Cd chemical shifts were observed as a function of both bile salt and $^{113}\text{Cd}^{2+}$ concentrations. A comparison between taurine conjugated tri- and dihydroxy bile salts was also made.

From a titration of the Na^+ -salt with $^{113}\text{Cd}^{2+}$, an apparent binding constant of 300 M^{-1} was obtained by fitting the experimental results to a simple equilibrium model for a 1:1 complex of Cd^{2+} -bile salt.

However, the observed chemical shift changes with the $^{113}\text{Cd}^{2+}$ concentration can not be fully described by this model. The observed ^{113}Cd NMR chemical shift could be fitted to a model consisting of 1:1 and 2:1 complexes of Cd^{2+} to taurocholate. The binding constants obtained were 1500 and 300 M^{-1} , respectively.

It has been suggested by Elgavish and Barnes (1983) from X-ray diffraction studies that Dy^{3+} and glycocholate (GC) form a 1:1 complex with an apparent binding constant K_a around 300 M^{-1} . The structure of this complex is such that Dy^{3+} is situated on the bisector of the O-C-O group which also means that the Dy^{3+} -hydroxyl distance is 10-14Å.

A structure for the Ca^{2+} -bile salt complex has been suggested by Moore (1984). A 1:1 complex between the bile salt and the Ca^{2+} ion is proposed where Ca^{2+} is bound inbetween the ionic head group and the hydroxyl groups on the steroid nucleus. An association constant for Ca^{2+} to monomeric taurocholate of 150 M^{-1} was reported. The association constant for calcium and micellar taurocholate was reported to be 7 M^{-1} . From what has been mentioned above it seems clear that more work is needed in order to understand the Ca^{2+} and bile salt interactions.

5.2. The ternary system sodium taurocholate-monoolein - heavy water

An important step in the study of complex systems is the establishment of the phase diagram. The complete determination of a phase diagram requires however very extensive work. The use of water deuterium NMR in distinguishing between different phases has been shown to be a very useful method.

In **Article VI** the phase diagram of sodium taurocholate (NaTC)-monoolein (MO) - heavy water (D_2O) is presented. The combination of NMR and polarized light microscopy studies show three one-phase regions: a homogeneous isotropic phase, a lamellar- and a cubic liquid crystalline phase. The phase diagram indicates that NaTC is a good solubilizer for MO. The isotropic phase appears already at a molar ratio of NaTC/MO of 0.4. The cubic liquid crystalline phase can not incorporate more than about 4% of NaTC before it is disrupted. The cubic structures seems to put rather strong conditions on the molecular packing and there are probably two causes for this disruption. One of these is the increased repulsion between the polar head groups as NaTC is included into the cubic phase. The other effect concerns the packing possibilities of the alkyl chains which are drastically changed when increasing the NaTC content. The lamellar liquid crystalline phase is stable up to about 55 % of water.

The lamellar liquid crystalline phase has also been studied with respect to hydration and counterion binding (**VI**). The water deuterium quadrupole splittings have been compared to a simple "two-site" model for the water molecules. The interpretation of the results according to this model lead to the conclusion that the hydration of the aggregate structure is maintained as the water content increases ("ideal swelling").

The lamellar liquid crystalline phase has also been studied using ²³Na quadrupole splittings as functions of the water content and the NaTC/MO molar ratios (**IV**). The results are compared to a theoretical calculation of fraction of sodium ions, at or near the aggregate surface

according to the Poisson-Boltzmann (P.B.) equation (Engström and Wennerström, 1978). At constant water content of 35w% the ^{23}Na quadrupole splittings were found to decrease with increasing NaTC to MO molar ratio. By combining the experimental results and the theoretical calculations the conclusion is that the decrease in the quadrupole splitting is mainly due to changes in counter ion location at the aggregate surface.

At a constant molar ratio of MO/NaTC = 0.2 the decrease in ^{23}Na quadrupole splittings as the water content increases is interpreted to be due to both a decrease in fraction of bound ions and to changes in the aggregate surface. This is, however, somewhat contradictory to the results from the water deuteron quadrupole splittings which suggested that the aggregate surface is invariant as the water content changes.

Many discussions in the bile salt area concern the size of the micelles. In **Article VI**, the ^1H FI-PGSE NMR method was used to determine self-diffusion coefficients for monomers and micelles. For the pure NaTC system the results indicate only minor changes in the size of the micelles with increasing concentration. This small increase probably depicts the gradual association behavior of the trihydroxy bile salts.

In the NaTC-MO system the mixed micelles were found to increase in size with increasing MO/NaTC molar ratio. A comparison between NaTC and MO self-diffusion coefficients suggests that at low MO/NaTC molar ratio (0.4) monomers, simple and mixed micelles are coexisting. At a higher molar ratio (0.8) only monomers and mixed micelles are present.

The NaTC-MO system was also studied by quasi-elastic light scattering (QLS) (**VI**). The combination of NMR and QLS revealed the coexistence of small micelles and vesicles. The formation of vesicles was confirmed using electron microscopy.

From dilution experiments using QLS the formation of vesicles was observed to occur very abruptly (VI). This behavior is quite different from the bile salt-lecithin system which shows a more gradual micelle-to-vesicle transition (Schurtenberger et al., 1985).

6. CONCLUDING REMARKS

The cation binding to two calcium binding proteins has been studied. The proteins were parvalbumin and bone γ -carboxyglutamic acid protein. In **Articles I-III** the cation binding to parvalbumin was investigated by means of ^{113}Cd NMR. Clear differences between α - and β -parvalbumins were obtained when the ^{113}Cd -loaded proteins were titrated with mono-, di- and trivalent ions.

In **Article IV** the calcium binding to the bone γ -carboxyglutamic acid protein from calf was studied using ^{43}Ca NMR. The results suggested that at least one Ca^{2+} ion may be bound to the protein with an binding constant in the range of $5 \cdot 10^3$ – 10^5 M^{-1} . Furthermore, the off-rate (k_{off}) for the calcium ion was found to be around $4 \cdot 10^3 \text{ s}^{-1}$.

The cation binding to a biological amphiphile, a bile salt, was studied in **Article IV** by means of ^{113}Cd NMR. The observed ^{113}Cd chemical shifts were found to fit well with a model consisting of both 1:1 and 2:1 complexes of Cd^{2+} to taurocholate.

The phase diagram of the sodium taurocholate (NaTC)-monoolein(MO)-water system has been outlined in **Article VI**. Three one-phase regions were found namely, an isotropic homogenous phase, a lamellar- and a cubic liquid crystalline phase. The sodium taurocholate is apparently a very good solubilizer for monoolein, a fact which is revealed through the large isotropic phase in the diagram.

Information about hydration and counterion binding in the lamellar phase was obtained from water deuteron and ^{23}Na -sodium NMR studies. From the water deuteron quadrupole splittings it is deduced that the hydration of the aggregate surface remains the same although the water content changes between 36-56w% D_2O . Sodium- 23 quadrupole splittings suggest, however, some changes in the aggregate surface over the same range of water content (VI).

Self-diffusion coefficients have been determined for the micelles in the sodium taurocholate-monoolein-water system (VI). The results indicate that monomers and mixed micelles coexist at a high MO/NaTC molar ratio (0.8). At a lower MO/NaTC ratio (0.4) monomers, simple and mixed micelles are coexisting.

From QLS measurements and electron microscopy the existence of vesicles in the NaTC-MO-water system was observed. By combining the results from NMR self-diffusion and QLS it was observed that when diluting a micellar sample, a two-phase region is reached between the lamellar liquid crystal-line- and the micellar phase.

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NMR studies on parvalbumin phylogeny and ionic interactions

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Summary

The inspection of several muscular parvalbumins from different species by two NMR methods (^{113}Cd resonance and ^1H relaxation measurements) allows two classes of parvalbumins to be distinguished according to their ion-binding properties. This result is in agreement with the phylogenetic classification of parvalbumins in two series, α and β , which was established on the basis of the primary structures of these proteins.

All parvalbumins are characterized by the presence of two primary cationic sites CD and EF, with structural features closely related to those already known on the basis of X-ray crystallographic studies of the β parvalbumin pI 4.25 from carp muscle.

However, parvalbumins of the β series are characterized by a secondary cation (Ca^{2+} , Mg^{2+} and other cations) binding site which is absent (or at least inaccessible) in parvalbumins of the α series.

The major component from thornback ray (pI 4.45) behaves as an α parvalbumin as shown by the present NMR studies, although its primary structure suggests a closer similarity with the parvalbumins of the β series.

Introduction

Parvalbumins or Pa form a class of homologous proteins of low molecular weight (about 12 000 daltons) which are present in abundance in the sarcoplasm of fast skeletal muscles of most vertebrates (1–4). These proteins are characterized by a high percentage of phenylalanine residues, an acidic isoelectric point (5) and by the strong binding of two calcium ions with K_{dCa} about 10^{-9} M (6, 7). The tertiary structure of one parvalbumin from carp muscle (component pI 4.25) has been established by X-ray crystallography using the calcium-loaded protein PaCa_2 (8, 9). The polypeptide chain is arranged in six helical fragments (denoted A–F) whose spatial organization delimits an internal core containing exclusively the side chains of non-polar residues such as Phe, Leu, Ile and Val. The two protein-bound calcium ions, which are separated

by 11.9 Å, are located in two well-defined sites with an octahedral arrangement of only oxygen-containing ligands. The two sites are termed CD and EF in reference to the helical fragments on each side of the calcium-binding domains.

Comparison between the amino acid sequences of parvalbumins from different species has indicated the occurrence of two distinct phylogenetic lineages α and β (10–12). Parvalbumins of both series are usually found in varied proportions in the muscles of a given species (5). In pike muscle the main component belongs to the α series (pI 5.0) and the minor component to the β series (pI 4.1), while in carp muscle the four major components belong to the β series and only one minor component belongs to the α series (10, 13). The major component (pI 4.45) of thornback ray cannot readily be classified as an α or β parvalbumin and it has been suggested that this protein is closer to the ancestral

protein from which the α and β lineages are derived (14).

The α and β parvalbumins differ by their isoelectric points (α parvalbumins are less acidic than β parvalbumins (1)) and by their immunological properties (15–17). It can be questioned whether α and β parvalbumins also differ in conformation. Only the tertiary structure of one β parvalbumin, i.e. carp 4.25, is presently known (8, 9). It is likely that α and β parvalbumins do not differ markedly in their tertiary structures as suggested by ^1H NMR studies (18). From a theoretical analysis of parvalbumin sequences it has been inferred that the six helical fragments A–F are likely to be conserved during evolution (19). Most of the non-polar residues forming the internal core of carp 4.25 parvalbumin are conserved in the sequences of α and β parvalbumins (11, 14). Furthermore, all the acidic residues Asp or Glu, which are involved in the coordination of the calcium ions in the CD and EF sites, are fully conserved, as are the residues Arg 75 and Glu 81, forming an internal salt bridge (11, 14). All these observations suggest that parvalbumins from both phylogenetic series α and β are characterized by very similar tertiary structures including the presence of the two cationic sites CD and EF.

^{113}Cd NMR spectroscopy has been shown to be well adapted to study the binding properties of the CD and EF sites of carp 4.25 parvalbumin (20, 21). Two distinct ^{113}Cd signals are observed for the cadmium-loaded species PaCd_2 which correspond to the $^{113}\text{Cd}^{2+}$ ions bound to the CD and EF sites of the protein. Proton relaxation enhancement (PRE) studies with the paramagnetic probe Mn^{2+} (22) as well as ^{25}Mg relaxation studies (21) have established the occurrence of a secondary site in carp 4.25 parvalbumin. In contrast to the primary sites CD and EF, this additional site exhibits no specificity among divalent cations. A similar affinity is observed for Ca^{2+} and Mg^{2+} ions (about 5×10^{-3} M) (22). The secondary site is most likely located close to the EF primary site and it was therefore named the 'EF satellite site' (22). Particularly relevant is the observation that the EF ^{113}Cd signal is very much dependent upon the addition of Mg^{2+} ions to carp 4.25 PaCd_2 while the CD ^{113}Cd signal remains unaffected (21).

In the present study two distinct NMR approaches, i.e. ^{113}Cd resonance measurements at

56.55 MHz and PRE T_1 measurements at 24 MHz, have been used to investigate the ion-binding properties of α and β parvalbumins comparatively. These studies include two typical α parvalbumins (pike 5.0 and rabbit 5.5) and five β parvalbumins (carp 3.95, carp 4.25, carp 4.47, hake 4.36 and pike 4.1) as well as the phylogenetically less well-defined parvalbumin pI 4.45 from thornback ray. The effects that divalent and monovalent ions have on the ^{113}Cd resonances of the parvalbumin-bound cadmium ions, as well as the PRE properties observed in the presence of the paramagnetic ions Mn^{2+} and Gd^{3+} afford a clear distinction between α and β parvalbumins on the basis of their ion-binding properties.

Materials and methods

Parvalbumins were isolated and used as lyophilized powders as previously described (2, 22, 23). Carps (*Cyprinus carpio*) were obtained at 'Domaine piscicole de Sylveréal', 30600 Vauvert (France). Hake (*Merluccius merluccius*) and thornback ray (*Raja clavata*) were obtained from local fisheries (Montpellier region). Pike (*Esox lucius*) and rabbit (*Oryctolagus cuniculus*) were obtained at the local market (Lund).

The purity of protein samples was checked by gel electrophoresis and by UV spectrometry. The calcium content was determined by atomic absorption spectrometry (values ranging from 2 to 2.5 Ca^{2+} ions per parvalbumin molecule). Parvalbumin components from a given species are named by their isoelectric points following the proposal by Pechère et al. (5) and their ionic content (PaCa_2 , $\text{PaCd}_{2,\dots}$, i.e. the two primary sites CD and EF filled with Ca^{2+} or Cd^{2+} ions respectively; PaCa_2Mg , $\text{PaCd}_2\text{Mn}_{\dots}$, i.e. the two primary sites CD and EF filled with Ca^{2+} or Cd^{2+} ions and the EF satellite site occupied by Mg^{2+} or Mn^{2+} ions). The concentration of parvalbumin solutions was determined by UV spectrometry following the relationship $\epsilon_{258} - \epsilon_{268} = 102 \times N$ (established in ref. 22, where N is the number of phenylalanine residues) for parvalbumins which only contain Phe residues (10 Phe for carp 4.25, carp 4.47 and hake 4.36; 9 Phe for pike 5.0 and rabbit 5.5) and from the absorbance at 258 nm using $\epsilon_{258} = 2900$ for carp 3.95 (9 Phe, 1 Tyr) and $\epsilon_{258} = 2700$ for thornback ray (8 Phe, 1 Tyr).

The ^{113}Cd NMR spectra were recorded at 56.55 MHz on a 6 Tesla spectrometer built at the Physical Chemistry Division (Lund). A 50 mM $\text{Cd}(\text{ClO}_4)_2$ solution containing Mn^{2+} (^{113}Cd natural abundance) was used as an external reference (positive chemical shifts to low field). The PRE T_1 measurements were performed at 24 MHz on a Bruker 302 S NMR spectrometer (Laboratoire de Chimie Structurale, Montpellier) as previously described (22). All reagents used in this study were of analytical grade. A $^{113}\text{CdSO}_4$ or $^{113}\text{Cd}(\text{ClO}_4)_2$ stock solution was prepared from ^{113}CdO (Oak Ridge, U.S.A.; 96.3% isotopic enrichment). Cadmium-loaded parvalbumins were prepared by chemical exchange of Ca^{2+} ions by Cd^{2+} ions added in small excess to PaCa_2 solutions (Cd^{2+} to PaCa_2 molar ratios between 2 and 2.5). Binding constant calculations were carried out using a minicomputer as described in (22). In this reference equation 3 shall be corrected for the missing term m_{1b} .

Results

^{113}Cd NMR studies

All parvalbumins studies are characterized by two ^{113}Cd resonances of nearly equal intensities between -80 ppm and -100 ppm (Fig. 1). This can be interpreted by the occurrence of two cationic sites CD and EF in agreement with previous results obtained with carp 4.25 parvalbumin (20). No clear distinction is apparent between parvalbumins from the α or β series on the basis of the chemical shifts of the two ^{113}Cd resonances (Table 1). In the case of β parvalbumins these resonances can be readily assigned to each of both sites CD and EF owing to the dependence of the EF chemical shift on the concentration of divalent cations, such as Mg^{2+} (21), or monovalent cations (see below). Since the cadmium-loaded parvalbumins were obtained by direct substitution of the bound calcium ions in PaCa_2 without any attempt to remove the Ca^{2+} and Cd^{2+} ions in excess, the chemical shifts reported in Table 1 are not intrinsic values for the $^{113}\text{Cd}^{2+}$ ions bound to the CD and EF sites. Ionic effects on ^{113}Cd chemical shifts are particularly significant in the case of β parvalbumins.

A clear distinction between α and β parvalbumins is obtained from the dependence of their

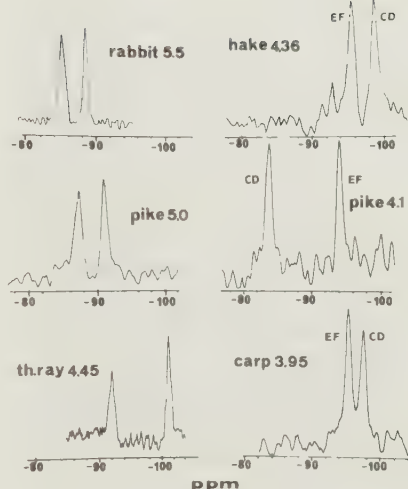


Fig. 1. ^{113}Cd NMR spectra at 56.55 MHz and 25 °C of several ^{113}Cd -loaded parvalbumins of the α series (left part) and the β series (right part) dissolved either in 50 mM Tris-sulphate at pH 7.5: hake 4.36 (8.2 mM), pike 5.0 (4.4 mM), pike 4.1 (3.1 mM) and carp 3.95 (6 mM) or in 50 mM cacodylate at pH 6.4: rabbit 5.5 (3.4 mM) and thornback ray 4.45 (1.9 mM). Usually the pulse angle was about 30° and the delay time 0.3–1 sec (1000–2000 scans).

Table 1. Chemical shifts in ppm of the CD and EF ^{113}Cd resonances in parvalbumins from the α and β series.

β series	EF signal	CD signal
Carp 4.25 (ref. 21)	-93.8	-97.5
carp 4.47	-93.0	-97.0
carp 3.95	-95.5	-97.8
Hake 4.36	-94.8	-98.3
Pike 4.1	-94.2	-83.8
α series	CD or EF signal	
Pike 5.0	-87.5 /	-90.2
Rabbit 5.5	-85.0 /	-88.6
Th. ray 4.45	-92.0 /	-101.

^{113}Cd resonances upon addition of divalent ions (Figs. 2-4) or monovalent ions (Figs. 5 and 6). As previously observed for carp 4.25 parvalbumin (21), all β parvalbumins studied here show a characteristic dependence of the chemical shift of one of the two ^{113}Cd resonances upon addition of Mg^{2+} ions (upfield variation of 3 to 6 ppm). This resonance is therefore assigned to the EF signal (see Table 1). Under the same conditions the CD ^{113}Cd signal remains practically unaffected.

In contrast, both ^{113}Cd resonances in the spectra of the α parvalbumins pike 5.0 (Fig. 2) and rabbit 5.5 (Fig. 3) remain practically unaffected upon Mg^{2+} addition. A similar result is observed for thornback ray 4.45 parvalbumin (Fig. 3). No decrease of the intensities of the ^{113}Cd signals is observed under the conditions of Figs. 2 and 3, even at very high Mg^{2+} concentrations (data not shown). This is in agreement with the fact that parvalbumins (α as well as β) have an affinity for Cd^{2+} ions higher than that for Mg^{2+} by about four orders of magnitude (22, 24).

When Ca^{2+} ions are added to carp 4.25 PaCd_2 the EF ^{113}Cd signal is shifted upfield (Fig. 4a) as is observed with Mg^{2+} ions. An apparent dissociation constant of about 2 mM is obtained from the

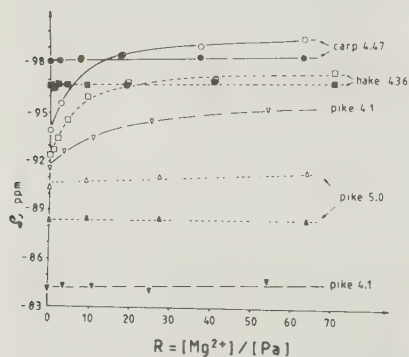


Fig. 2. Chemical shift dependence of the ^{113}Cd resonances of the four parvalbumins, carp 4.47, pike 4.36, pike 4.1 and pike 5.0, on MgSO_4 concentration. Buffer conditions: 50 mM Tris-sulphate at pH 7.5. The chemical shifts δ (ppm) are plotted versus $R = [\text{Mg}^{2+}]/[\text{Pa}]$. The samples were previously used for the ammonium titrations described in Fig. 6 and therefore contain an excess of ammonium sulphate.

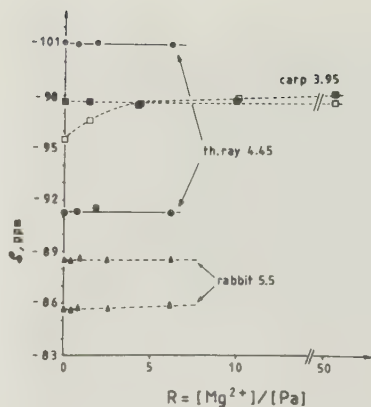


Fig. 3. Chemical shift dependence of the ^{113}Cd resonances of the three parvalbumins (carp 3.95, rabbit 5.5 and thornback ray 4.45) on MgSO_4 concentration. The chemical shifts δ (ppm) are plotted versus $R = [\text{Mg}^{2+}]/[\text{Pa}]$. Buffer conditions as in Fig. 1. No ammonium sulphate present.

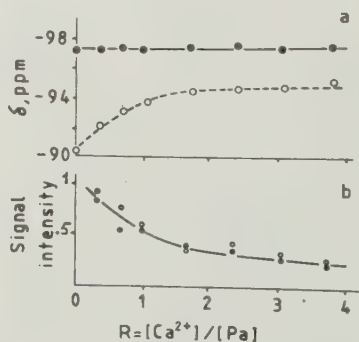


Fig. 4. Dependence of the ^{113}Cd resonances of carp 4.25 parvalbumin (8.5 mM) on $\text{Ca}(\text{ClO}_4)_2$ concentration: a: the chemical shifts δ (ppm) are plotted versus $R = [\text{Ca}^{2+}]/[\text{Pa}]$. b: the relative intensities of the two signals are plotted versus R . Tris-sulphate buffer 50 mM at pH 7.5. (●) CD signal and (○) EF signal.

chemical shift variation as a function of Mg^{2+} concentration observed with the EF ^{113}Cd signal. The chemical shift effects could only be followed up to Ca^{2+} to protein molar ratios of about 5 since the intensities of the CD and EF ^{113}Cd signals decrease when the Ca^{2+} concentration increases as a result of Cd^{2+} substitution by Ca^{2+} in the CD and EF sites. It can be seen in Fig. 4b that both protein-bound Cd^{2+} ions are readily substituted by Ca^{2+} ions. This observation is in agreement with the fact that carp 4.25 parvalbumin has only a slightly higher affinity for Cd^{2+} ions than for Ca^{2+} ions (22). An upfield shift of the EF ^{113}Cd signal is also observed when Cd^{2+} ions are added to carp 4.25 PaCd₂ (data not shown).

In contrast to the divalent cations considered above, the monovalent cations Na^+ and NH_4^+ show a selective downfield variation by about 1–2 ppm of the EF ^{113}Cd signal of β parvalbumins, while the CD ^{113}Cd signal remains practically unaffected (Figs. 5 and 6). An apparent binding constant of about 10^{-1} is calculated from the dependence of the chemical shift of the EF ^{113}Cd signal on Na^+ concentration (Fig. 5). For the α pike 5.0 parvalbumin both ^{113}Cd signals remain unaffected upon addition of NH_4^+ ions (Fig. 6) and this is also the case for th. ray 4.45 parvalbumin. The downfield effect produced by NH_4^+ ions for the β parvalbumins is readily reverted by addition of Mg^{2+} ions. This is shown by the data

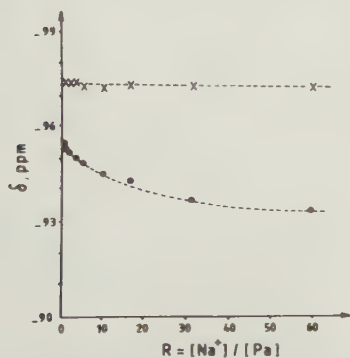


Fig. 5. Chemical shift dependence of the ^{113}Cd resonances of carp 4.25 parvalbumin (6.4 mM) on NaCl concentration. Buffer conditions: 50 mM cacodylate at pH 6.4.

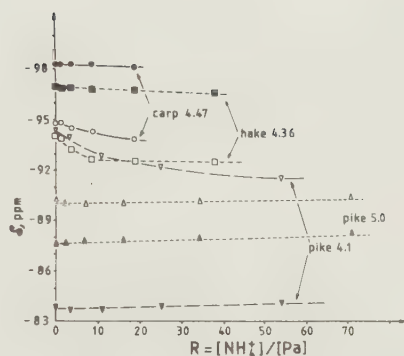


Fig. 6. Chemical shift dependence of the ^{113}Cd resonances of the four parvalbumins carp 4.47, hake 4.36, pike 4.1 and pike 5.0 on $(\text{NH}_4)_2\text{SO}_4$ concentration. Buffer conditions: 50 mM Tris-sulphate at pH 7.5. The chemical shifts δ (ppm) are plotted versus $R = [\text{NH}_4^+] / [\text{Pa}]$.

obtained with three β parvalbumins, i.e. carp 4.47, hake 4.36 and pike 4.1, which were first titrated by NH_4^+ ions (Fig. 6) and afterwards by Mg^{2+} ions (Fig. 2). By way of comparison the effect due to the single addition of Mg^{2+} ions is shown in Fig. 3 for the β carp 3.95 parvalbumin (see also ref. 21 for carp 4.25).

PRE studies (T_1 measurements)

A previous PRE study with the β carp 4.25 parvalbumin showed that Gd^{3+} is a selective probe for the primary sites CD and EF, while Mn^{2+} acts as a probe for the EF satellite site of this parvalbumin (22). The α pike 5.0 parvalbumin has been studied here under very similar conditions. A striking difference is observed between the two phylogenetically distinct proteins in the presence of Mn^{2+} . Fig. 7 shows the variation of the enhancement factor ϵ (for a definition see ref. 31, chap. 11) when a 0.5×10^{-4} M Mn^{2+} solution is titrated at increasing PaCa_2 concentrations for the β carp 4.25 parvalbumin (data adapted from ref. 22) and for the α pike 5.0 parvalbumin. The latter is characterized by much lower ϵ values. The theoretical curve ϵ v. $[\text{PaCa}_2]$ for pike 5.0 parvalbumin is drawn from $K_{d\text{Mn}} = 2 \times 10^{-8}$ M and $\epsilon_b = 3.5$ (the intrinsic

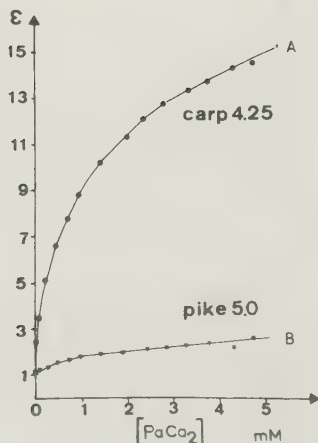


Fig. 7. Protein-titrations at 25 °C of 0.5×10^{-4} M MnSO_4 in 50 mM Na-cacodylate buffer at pH 6.0 using the PRE method (proton T_1 measurements at 24 MHz). The water proton relaxation enhancement ϵ is plotted versus PaCa_2 : (a) carp 4.25 parvalbumin from the β series (adapted from ref. 22) and (b) pike 5.0 parvalbumin from the α series (this work). The theoretical curves are calculated for $n = 1$ (stoichiometric ratio), $K_{d\text{Mn}} = 10^{-3}$ M and $\epsilon_b = 17$ (intrinsic enhancement in the case of carp 4.25 parvalbumin) and for $n = 2$, $K_{d\text{Ca}} = 10^{-9}$ M, $K_{d\text{Mn}} = 2 \times 10^{-8}$ M and $\epsilon_b = 3.5$.

enhancement factor) assuming $K_{d\text{Ca}} = 10^{-9}$ M (7) and competition for two equivalent sites in accordance with data of ref. (24). Table 2 gives a comparison between PRE data obtained in this work with α pike 5.0 parvalbumin and those previously reported (22) for β carp 4.25 parvalbumin.

The affinity of the monovalent ions Na^+ and NH_4^+ for the EF satellite site of carp 4.25 parvalbumin was studied by competition with the Mn-loaded PaCa_2 molecule as shown in Fig. 8. In contrast to divalent cations which have an association constant about 10^3 M^{-1} (22), these monovalent cations bind to the secondary site with an affinity at least ten-fold lower, which is in agreement with the above-mentioned ^{113}Cd NMR experiments. The decrease in ϵ at increasing Na^+ or NH_4^+ concentration does not follow a strict competition pattern as shown by the difference between the experimental

Table 2. Intrinsic enhancement factors ϵ_b determined at 24 MHz and 25 °C.

parvalbumin	$\epsilon_b (\text{Gd}^{3+})$	$\epsilon_b (\text{Mn}^{2+})$
α pike 5.0 ^a	2.5	3.5
β carp 4.5 ^b	2.3	17

^a This work

^b Values from ref. 22

curve and the theoretical curve calculated for $n = 1$, $K_{d\text{Mn}} = 0.6$ mM and $K_{d\text{Na}} = K_{d\text{NH}_4} = 70$ mM (Fig. 8). The residual enhancement observed at high ionic concentration is likely to be due to a partial binding of Mn^{2+} to the primary sites CD and EF. These sites are characterized by a much lower ϵ_b value than the EF satellite site and therefore their contribution to the observed relaxation enhancement is only apparent when the occupancy of the secondary site is sufficiently low. In agreement with this interpretation the residual enhancement due to Mn^{2+} ions bound to the parvalbumin molecule is suppressed on addition of La^{3+} ions as a consequence of the selective occupation of the primary sites CD and EF by the trivalent lanthanide ion, as shown in Fig. 8.

^{113}Cd NMR study in the presence of Mn^{2+}

Since it is well established that Mn^{2+} interacts

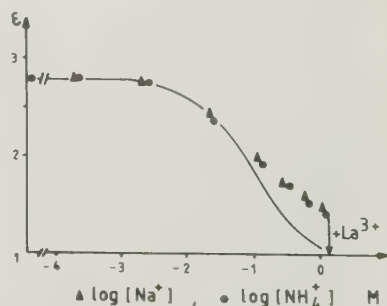


Fig. 8. Competition of Na^+ and NH_4^+ with Mn^{2+} (3×10^{-4} M) for carp 4.25 PaCa_2 (1×10^{-4} M), ϵ v. $\log [\text{Na}^+]$ or $\log [\text{NH}_4^+]$. A theoretical curve is calculated for $n = 1$, $K_{d\text{Na}} = K_{d\text{NH}_4} = 0.07$ M and $\epsilon_b = 17$. The residual enhancement decreases to $\delta = 1$ upon addition of 10 eqs La^{3+} (excess) as already described in ref. 22.

with the secondary site of carp 4.25 parvalbumin (see ref. 22 and above), a ^{113}Cd NMR experiment was undertaken in order to evaluate the distance between this secondary site and the CD and EF primary sites of the protein. As shown in Fig. 9, the EF ^{113}Cd signal is strongly broadened when Mn^{2+} is added to PaCd_2 . In the Mn^{2+} concentration range used in this experiment the half-width of the signal increases linearly as the Mn^{2+} concentration is increased. In contrast, the CD signal remains practically unaffected by the addition of Mn^{2+} ions (not shown). If one assumes that the observed broadening is due to the dipolar interaction between the Mn^{2+} electronic spin and the ^{113}Cd nuclear spin, so that the half-width of the nuclear signal is proportional to the inverse sixth power of the electron-nucleus distance according to the Solomon-Bloembergen equation (31), it follows that the paramagnetic center Mn^{2+} is located close to the EF site (5 Å or less) and far away from the CD site (9–10 Å or more). It is therefore justified, as previously proposed (22), to give the name of EF

satellite site to the cationic secondary site of carp 4.25 parvalbumin owing to its proximity to the EF primary site. The determination of an accurate value of the distance between both sites requires a more extended study than reported in Fig. 9 (work in progress).

Discussion

The results obtained by ^{113}Cd NMR with parvalbumins from both phylogenetic lineages α and β (Fig. 1 and Table 1) establish that the occurrence of two cationic sites CD and EF is a general feature of parvalbumins. It should be noted that in addition to PaCd_2 , the fully cadmium-loaded protein, hybrid species such as PaCaCd and/or PaCdCa (i.e. with one of the two primary sites filled with Ca^{2+} and the other with Cd^{2+}) are likely to be present in solution when a slight excess of Cd^{2+} ions is added to PaCa_2 . However, the conclusions of the present study will not be affected by this multiplicity, provided the observed ^{113}Cd resonances are associated with the binding of Cd^{2+} to the CD and EF sites of the parvalbumin molecule. As previously discussed (21), it is likely that the hybrid Ca-Cd species have ^{113}Cd chemical shifts which are undistinguishable from those of the PaCd_2 species.

Because of the high-field position of the ^{113}Cd resonances (between -80 ppm and -100 ppm) it can be concluded that the CD and EF sites of parvalbumins (α as well as β) include only oxygenated ligands, in agreement with the strict conservation of the acidic residues Asp and Glu involved in cation binding (11). Other calciproteins, for which CD or EF domains have been recognized in their primary structures, are also characterized by ^{113}Cd chemical shifts close to those observed for parvalbumins. Calmodulin and troponin C give rise to resonances in the chemical shift ranges, -85 to -120 ppm (25) and -107 to -110 ppm (26), respectively. The assignment of the two ^{113}Cd signals of parvalbumins cannot be obtained on the basis of their chemical shifts. Although the CD signal of β parvalbumins usually occurs at higher field positions than the EF signal the reverse situation is observed for the β pike 4.1 parvalbumin (see Fig. 1). An unambiguous assignment is achieved with the β parvalbumins for which the EF signal shows a selective dependence on ionic strength, as previ-

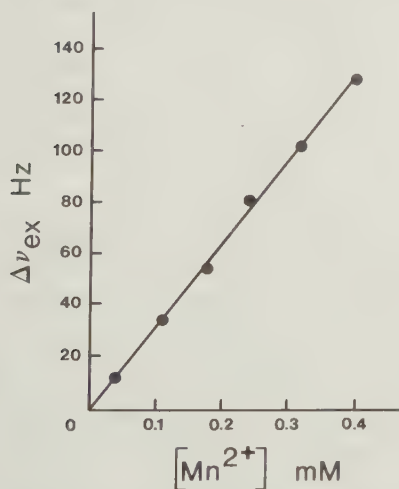


Fig. 9. Linewidth dependence of the ^{113}Cd NMR signal at -93.8 ppm (EF signal) of carp 4.25 PaCd_2 upon Mn^{2+} concentration. Protein concentration about 5 mM. The excess linewidth at half maximal height $\Delta\nu_{\text{ex}} = \Delta\nu_{\text{obs}} - \Delta\nu_{\text{Mn free}}$ is plotted versus $[\text{Mn}^{2+}]$.

ously observed for carp 4.25 parvalbumin (21). Since this criterion does not apply for α parvalbumins, no attempt was made to assign the ^{113}Cd resonances of these proteins on the basis of their chemical shifts. However, differences in the relaxation properties of the two signals may eventually provide a means of identifying the CD and EF resonances in all parvalbumins. We have observed that the longitudinal relaxation time T_1 (measured by the inversion-recovery technique) of the EF ^{113}Cd signal is shorter than that of the CD signal in carp 4.25 parvalbumin (0.3 s and 0.7 s, respectively). This certainly reflects the differences in the coordination of the two sites (27). The relative sharpness of the CD and EF ^{113}Cd resonances (half-widths ca. 40 Hz) is likely to be related to slow kinetics of cation exchange between the CD or the EF site and the external medium. This is in agreement with previous results with ^{43}Ca NMR which indicated that the life-time of Ca^{2+} in PaCa_2 is much larger than 1 ms at ambient temperature (28).

The selective effect on the EF ^{113}Cd resonance observed upon addition of divalent cations (Mg^{2+} , Ca^{2+} and Cd^{2+}) or monovalent cations (Na^+ and NH_4^+) to several β parvalbumins suggests that parvalbumins of this phylogenetic series are characterized by the presence of a secondary site as previously discussed for carp 4.25 parvalbumin (21). Apparent affinity constants deduced from the ^{113}Cd NMR results in Figs. 2-6 have values about 10^3 M^{-1} , while for Na^+ and NH_4^+ they are lower by at least one order of magnitude, in agreement with the parallel PRE studies reported in the section Results. It therefore appears that the dependence of the EF ^{113}Cd signal on the concentration of monovalent or divalent cations for parvalbumins of the β series is related to the binding of these ions to a secondary site as previously discussed with carp 4.25 parvalbumin (21).

According to the results obtained with two typical α parvalbumins, i.e. pike 5.0 and rabbit 5.5, for which no dependence of the ^{113}Cd chemical shifts is observed upon addition of monovalent or divalent cations (see Figs. 2, 3 and 6), it is reasonable to conclude that no EF satellite site is present in the α series, or at least that such site is not accessible. The low ϵ_b value observed by PRE for the binding of Mn^{2+} to pike 5.0 parvalbumin (see Table 2) is consistent with the exclusive binding of

Mn^{2+} to the CD and EF primary sites of this α parvalbumin and with the absence of any secondary binding. A similar ϵ_b value is found when Gd^{3+} binds to the CD and EF sites. This suggests that the primary sites of pike 5.0 are highly dehydrated, as in the case of carp 4.25 parvalbumin.

The present structural studies allow parvalbumins to be classified into two groups: the one is characterized by the occurrence of a Ca-Mg secondary site and the other is devoid of such a site. As discussed below this distinction probably reflects a difference in the tertiary structures of these two groups of parvalbumins and it coincides with the classification (α and β lineages) previously inferred from the comparison of the amino acid sequences of a large number of parvalbumins. All β parvalbumins examined here (i.e., carp 4.25, carp 4.47, hake 4.36 and pike 4.1) are characterized by the occurrence of a Ca-Mg secondary site while the two α parvalbumins examined (i.e. pike 5.0 and rabbit 5.5) apparently have no secondary site. However, more examples need to be considered in order to establish that there is a strict correspondence between the classification based on primary structure analysis and the classification based on tertiary structure analysis. It must be noted that the latter classification does not require the sequence to be known and therefore a rapid screening of as many parvalbumins as are available is possible by using the NMR techniques. In this respect carp 3.95 parvalbumins, whose sequence is not established, can be readily classified as a β parvalbumin as shown by ^{113}Cd NMR (see Fig. 3).

The phylogenetic studies based on amino acid sequences suggest that parvalbumins derive from an ancestral sequence composed of four polypeptide domains (labelled I-IV) of nearly equal length (about 40 amino acids) which appear to be common to many calcium-binding proteins (11, 12). These domains are structurally related to the CD and EF domains of carp 4.25 parvalbumin, each of which consists of two helices and a calcium-binding loop in between, as established by X-ray crystallography (8, 9). According to Goodman et al. (12) it can be assumed that the primitive I-II-III-IV polypeptide structure has evolved in the parvalbumin lineage to give rise to a II-III-IV structure with the loss of the calciumbinding function of domain I.

An attractive possibility would be that the second-

secondary site found in β parvalbumins is located within domain II. However, examination of the presently known amino acid sequences of β parvalbumins shows that domain II is not systematically substituted with acidic residues in positions favourable for calcium-binding to occur. Furthermore, cation binding to domain II would involve distances between the secondary site and both primary sites CD and EF larger than 10 Å, a fact which is not compatible with the ^{113}Cd NMR study described in Fig. 9. The selective broadening of the ^{113}Cd signal upon Mn^{2+} addition to carp 4.25 PaCd₂ clearly indicates that the Mn^{2+} ion bound to the secondary site of the protein is distant by no more than 5 Å from the EF primary site. It has also been determined that about three water molecules remain attached to the protein-bound Mn^{2+} ion (Parello et al., unpublished PRE results). In relation to this relatively high hydration, the secondary site of carp 4.25 parvalbumin is characterized by a high intrinsic water proton enhancement, i.e. $\epsilon_b = 17$ (see Table 2), when Mn^{2+} binds to PaCa₂ to form the hybrid species PaCa₂Mn. As illustrated in Fig. 10 the most likely location of the secondary site is at the periphery of the protein molecule close to the EF primary site.

The occurrence of an additional site in carp 4.25 parvalbumin appears to be somewhat controversial. Nelson et al. (29) proposed that, in addition to the binding to both CD and EF primary sites, the lanthanide ion Tb^{3+} is capable of binding to a hydrated third site thus explaining the fluorescence behaviour observed when PaCa₂ is titrated with Tb^{3+} ions (biphasic curve: fluorescence enhancement at low binding ratios followed by a fluorescence decrease at higher binding ratios). It might be that this hydrated site is identical to the EF satellite site inferred from NMR studies (21, 22). However, biphasic fluorescence curves are observed with parvalbumins of the β series as well as with parvalbumins of the α series, although the latter have apparently no EF satellite site (Cavé, unpublished results). Furthermore, as indicated in Table 2, only very low ϵ_b values compatible with low hydration are observed when Gd^{3+} binds to carp 4.25 or pike 5.0 parvalbumins.

An alternative explanation given by Sodawski et al. (30) suggests that a fluorescence biphasic curve can be predicted on the basis of sequential binding of Tb^{3+} to the CD and EF primary sites without any need of an additional site. However, it has been observed by one of us (A.C.) that addition of

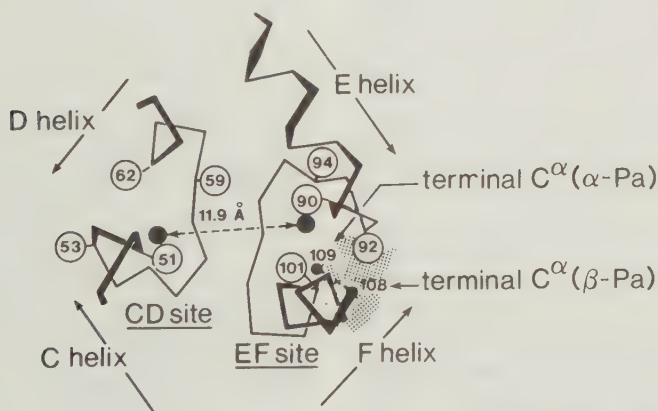


Fig. 10. Schematic representation of the CD and EF calcium-binding sites of carp 4.25 parvalbumin according to ref. 32. The EF satellite site (Ca-Mg secondary site) is tentatively located at the hinge between helices E and F and the EF loop (dashed area). The F helix is tentatively prolonged by one residue in α parvalbumins. Circled numbers correspond to the sequence positions occupied by Asp or Glu residues involved in the coordination of the CD and EF Ca^{2+} ions (□).

paramagnetic Gd^{3+} ions to cadmium-loaded parvalbumins either from the α series or the β series induces a selective broadening of one of the two ^{113}Cd NMR signals concomitantly with a reduction of their intensities. This observation differs from a previous report (20). This would suggest that an additional Gd^{3+} binding occurs besides the substitution of Cd^{2+} by the lanthanide ion in the CD and EF primary sites in both parvalbumin series. Obviously, the binding of lanthanides to parvalbumins follows a rather complex process which is not fully understood. However, the inference of a secondary site in carp 4.25 parvalbumin by PRE and ^{113}Cd NMR (21, 22) appears to be less problematical.

It was previously observed that addition of a trivalent lanthanide Ln^{3+} ion to $PaCa_2Mn$ is accompanied by a total release of Mn^{2+} when the Ln^{3+}/Pa molar ratio is increased up to about 2 (22). It is likely that the observed release of Mn^{2+} from the secondary site occurs as a consequence of the filling of the EF primary site with Ln^{3+} ions. A possible explanation for this binding dependence is that one (or several) ligand(s) is (are) shared by the two cationic sites closely located in space (the EF primary site and the EF satellite site, respectively). The simultaneous binding of two close divalent cations, as is found in the hybrid species $PaCa_2Mg$, $PaCa_2Mn$, $PaCd_2Mg$ would be allowed on an electrostatic basis, while the presence of a trivalent cation in the EF primary site would prevent any additional divalent cation to bind to the secondary site.

As shown in Fig. 10 the dotted area in the EF domain appears to be a suitable location for the secondary site of carp 4.25 parvalbumin, if one considers the density of carboxylic groups available for cation coordination. Two types of carboxylate groups are found in this area: one involving amino acids which participate to the coordination of the EF Ca^{2+} ions (Asp-90, Asp-92, Asp-94 and Glu-101) and the other one involving a free carboxylic group (Asp-100). Some of these carboxylate groups might be common to the EF primary site and the secondary site. According to X-ray crystallography residues 92 and 101 of carp 4.25 $PaCa_2$ coordinate with both oxygen atoms of their carboxylate groups (32). The presence of acidic residues at positions 90, 92, 94, 100 and 101 is a common feature of parvalbumins from both α and β series (see ref. 11),

and it is not obvious on the basis of their primary structures to point out which is the crucial difference that allows the additional cation binding to occur in β parvalbumins. Other regions of the primary structures, which are characterized by a cluster of acidic residues, are also common to both phylogenetic series. Although α parvalbumins have higher pI values, it appears that their content in aspartyl and glutamyl residues exceeds by about three residues that are found in β parvalbumins (11). It is likely that the observed structural difference between α and β parvalbumins is to be found within the tertiary structure organization of these two groups of proteins. It might be that in α parvalbumins no additional cation binding occurs, besides that involving the CD and EF primary sites, because of an unfavourable spatial arrangement of some of the acidic side chains. An interesting observation in this respect is that the F domain in the C-terminal part of the polypeptide chain of parvalbumins is systematically longer by one residue in the α series than in the β series (11). It is likely that the helical conformation of the F domain will be conserved in all parvalbumins. The C-terminal carboxylic group will therefore occupy quite different spatial positions in the two parvalbumin series. Inspection of the three-dimensional model of carp 4.25 parvalbumin, inferred from X-ray crystallography, indicates that the α -carbon of the last residue will be more 'internal' in the α series in comparison to the β series (see Fig. 10). Although the distant terminal carboxylic group is not to be retained as a direct ligand for cation coordination it might nevertheless play a role in cation binding to the secondary site by acting on the position of the F helix relative to the rest of the protein structure. Since the secondary site is tentatively located at the hinge between the F helix (and/or E helix) and the EF loop (see Fig. 10) the positioning of the F helix would be crucial for the structural organization of the ligands in the secondary site. Obviously, it remains to be unambiguously established which are the protein side chains involved in this site before a structural distinction can be clearly delineated between α and β parvalbumins.

It is interesting to note that ray 4.45 parvalbumin behaves as an α parvalbumin, since no dependence of either ^{113}Cd signal is observed on the ^{113}Cd NMR spectrum of $PaCd_2$ upon addition of Mg^{2+} as shown in Fig. 3. However, in their phylogenetic

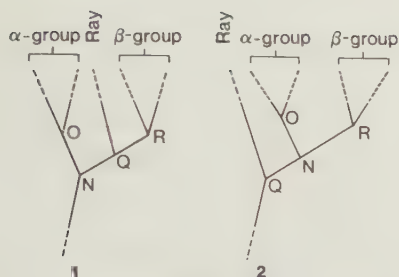


Fig. 11. Alternative dendrograms 1 and 2 in the parvalbumin evolutionary tree differing by the positions of the ray lineage (according to ref. 11).

studies of parvalbumins using the maximum parsimony method (i.e. lowest sum of nucleotide replacements and gene duplicative events) Goodman and Pechere noted that to place the ray lineage with the β -group (dendrogram 1) is nearly as parsimonious as to have it emerge from the stem to a common $\alpha\beta$ ancestor (dendrogram 2) but to place it with the α -group is clearly less parsimonious (11). Studies of parvalbumin evolution based on ten parvalbumin sequences, as well as on the sequences of related calcium-binding proteins, thus allowed two alternative genealogical trees to be established, which differ primarily in the positions of the ray lineage (dendrograms of type 1 and 2 respectively, as shown in Fig. 11). Analysis of a larger set of sequences of parvalbumins and calcium-binding proteins recently allowed a detailed evolutionary tree of the calcium-binding protein family to be established (12). The parvalbumin part of this tree, as shown in Fig. 12, favours a dendrogram of type 1 with the ray lineage closely related to the β -group. However, as far as the NMR evidence is considered, it is reasonable to conclude that ray 4.45 parvalbumin does not belong to the β -group.

The present NMR studies clearly establish that the occurrence of a Ca-Mg secondary site is a structural feature common to hake 4.36, pike 4.1, carp 4.47 and carp 4.25 parvalbumins. On the basis of the genealogical tree of Fig. 12 it can be stated that the emergence of this additional cation binding occurred at node T or before it. We had no opportunity to investigate other β parvalbumins by NMR, in particular frog 4.5 and coelacanth 4.5, in order to establish if the occurrence of the Ca-Mg

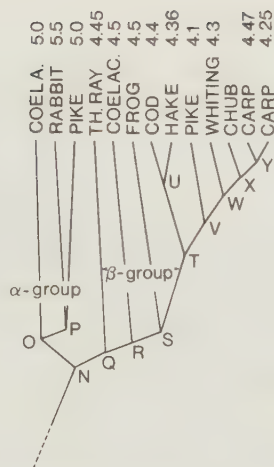


Fig. 12. Parvalbumin branching-off in the evolutionary tree of the calcium-binding protein family (according to ref. 12).

secondary site is a structural feature of all parvalbumins emerging from node R in the evolutionary tree (Fig. 12).

It must be noted that the correspondence between the two classifications, one obtained on the basis of primary structure and the other obtained on the basis of tertiary structure, is remarkable although there is no obvious explanation why the differences in amino acid composition and sequence between α and β parvalbumins would give rise to the structural difference observed, i.e. the occurrence of a Ca-Mg secondary site in β parvalbumins in contrast to α parvalbumins. The understanding of such a structural difference would require a detailed knowledge of the tertiary structure of a typical α parvalbumin, which is not presently available. It must be emphasized that the presence of a secondary site in parvalbumins might be only one among other structural differences between the two parvalbumin lineages. The NMR techniques presented in this work (PRE measurements; ^{113}Cd resonance) are well adapted to extend the phylogenetic classification to parvalbumins of unknown primary structure.

Acknowledgements

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Metal-ion binding to parvalbumin

A ^{113}Cd -n.m.r. study of the binding of different lanthanide ions

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^{113}Cd -n.m.r. studies were used to investigate the binding of the lanthanide ions La^{3+} , Gd^{3+} , Tb^{3+} , Yb^{3+} and Lu^{3+} to parvalbumins. It was shown that lanthanide ions with a smaller ionic radius bind sequentially to Cd^{2+} -saturated parvalbumin, whereas those with a larger ionic radius bind with similar affinity to both the CD site and the EF site. The smallest ion, Lu^{3+} , does in fact not compete significantly with Cd^{2+} for the CD site in carp parvalbumin, but appears to bind only to the EF site. This preference of the smaller lanthanide ions for the EF site was used to assign the n.m.r. signals for protein-bound ^{113}Cd . By using Cd n.m.r. and Tb^{3+} fluorescence it was also shown for α -lineage parvalbumin from pike that these proteins possess a third site that can bind lanthanide ions. This site is, however, much weaker than in the β -lineage parvalbumins. It was used to assign the ^{113}Cd resonances from protein-bound Cd^{2+} ions in the spectrum of pike pI5.0 parvalbumin.

X-ray-crystallographic studies performed on carp pI4.25 parvalbumin have elucidated the tertiary structure of this protein (Kretsinger & Nockolds, 1973; Coffee *et al.*, 1972). These studies showed that two primary sites, designated CD and EF, are symmetrical and that they co-ordinate Ca^{2+} ions through oxygen ligands from four carboxylic groups and one peptide carbonyl group. The sixth ligand is provided by a serine-residue side chain for the CD site and by a water molecule for the EF site. Kretsinger (1976) has pointed out that the EF site should be considered as eight-co-ordinated, since two of the carboxylate groups participate with both oxygen atoms in the co-ordination. In contrast, the CD site is six-co-ordinated. The X-ray studies have also shown that Tb^{3+} ions have a preference for the EF site over the CD site (Moevs & Kretsinger, 1975; Sowadski *et al.*, 1978). Furthermore, Lee & Sykes (1981) have observed that Yb^{3+} ions bind sequentially to the primary sites of the Ca^{2+} -loaded carp pI4.25 parvalbumin. They proposed that the EF site was the first one to be saturated by Yb^{3+} ions, in analogy with the X-ray studies.

The X-ray studies were not able to characterize

an external, secondary, site that was evidenced by optical studies with Tb^{3+} and Eu^{3+} as luminescence probes (Nelson *et al.*, 1977; Horrocks *et al.*, 1980; Horrocks & Collier, 1981).

Previous ^{113}Cd -n.m.r. studies of metal-ion binding to parvalbumins have revealed that the two sites give rise to separate ^{113}Cd -n.m.r. signals with shifts between -80 and -100 p.p.m. (Drakenberg *et al.*, 1978; Cavé *et al.*, 1982). It therefore appeared possible to use ^{113}Cd -n.m.r. in order to study selectivity of lanthanide-ion binding to the CD and EF sites. In the present paper we describe such studies on carp pI4.25 parvalbumin, belonging to the β -lineage, and pike pI5.0 parvalbumin, belonging to the α -lineage of the parvalbumin family. These two proteins have the same co-ordinating groups in the primary metal-ion-binding sites. However, thus far only the parvalbumins belonging to the β -phylogenetic lineage have been shown to contain the third, secondary, site, which is located close to one of the two primary sites (Cavé *et al.*, 1982). We also present evidence for the existence of this site in parvalbumins belonging to the α -phylogenetic lineage. This has been of help in identifying the CD and EF sites in ^{113}Cd -n.m.r. spectra of the α -parvalbumins as we have previously shown for the β -parvalbumins (Cavé *et al.*, 1982).

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Materials and methods

Carp p14.25 parvalbumin and pike p15.0 parvalbumin were prepared as described by Cavé *et al.* (1982) and stored as freeze-dried powder until use. Calcium was removed from the parvalbumins by using the Chelex procedure (Wolff *et al.*, 1977). Cadmium was added from a stock solution of $^{113}\text{Cd}(\text{ClO}_4)_2$ prepared from ^{113}CdO (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) with an isotopic enrichment of 96%. The concentrations of the parvalbumin solutions (1–3 mM) were determined by u.v. spectroscopy by using the relationship $\epsilon_{258} - \epsilon_{268} = 102 N \text{ M}^{-1} \text{ cm}^{-1}$, where N is the number of phenylalanine residues in the protein molecule (Cavé *et al.*, 1979a).

The ^{113}Cd -n.m.r. spectra were recorded on a laboratory-built UL-6T spectrometer (Drakenberg *et al.*, 1983). Standard acquisition parameters were: flip angle 45° (8 μs), pulse delay 0.6 s, spectral width 20000 Hz, number of real data points 8000 and number of transients 25000–100000. The ^{113}Cd -n.m.r. chemical shifts are given relative to the shift of an aqueous 0.1 M- $\text{Cd}(\text{ClO}_4)_2$ solution, with high-field shifts given negative values.

Results

^{113}Cd -n.m.r. study of carp parvalbumin (p14.25)

^{113}Cd -n.m.r. spectra of Cd^{2+} -saturated parvalbumins display two resonances, one from the CD site and the other from the EF site (Drakenberg *et al.*, 1978). When the $^{113}\text{Cd}^{2+}$ -saturated protein is titrated with various lanthanide ions (La^{3+} , Gd^{3+} , Tb^{3+} , Yb^{3+} and Lu^{3+}) the lanthanide-ion competition appears clearly as decreases in the intensities of the ^{113}Cd -n.m.r. signals.

Fig. 1 depicts the decrease in the intensities of the two original ^{113}Cd resonances, 1 and 2 (arbitrarily labelled from low to high field), as well as the variation in intensities of novel resonances for protein-bound $^{113}\text{Cd}^{2+}$ as a function of added equivalents of lanthanides (R). La^{3+} , the largest ($R_0 = 0.115 \text{ nm}$), and Lu^{3+} , the smallest ($R_0 = 0.093 \text{ nm}$), are both diamagnetic, and thus the interpretation of results obtained with these two ions is not complicated by paramagnetic effects, as it is for the other lanthanide ions. Titration with La^{3+} results in a simultaneous decrease in the intensities of both resonances. A new resonance labelled 2' appears in parallel with the resonance from free Cd^{2+} ions. Resonance 2' has a shift of -100 p.p.m. , which, like the shift of signal 2, does not vary with La^{3+} concentration and is therefore assigned to the same site as resonance 2. The shift of resonance 1 depends on the La^{3+} concentration and has shifted upfield by approx. 4 p.p.m. before the signal intensity be-

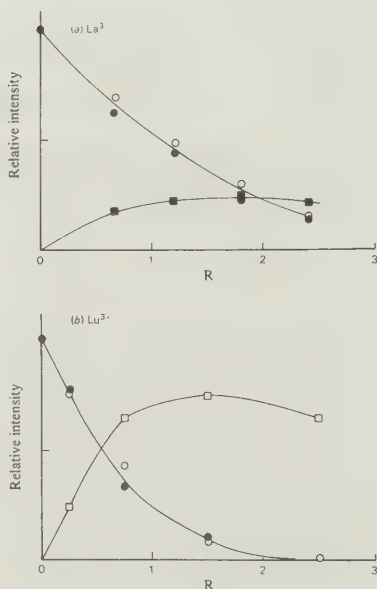


Fig. 1. Relative intensities of the ^{113}Cd -n.m.r. resonances from Cd^{2+} bound to carp (p1425) parvalbumin as a function of added La^{3+} or Lu^{3+} .

The solution contained 2 mM- PaCd_2 in 50 mM-sodium cacodylate buffer, pH 6.1. $\circ$, Site 1 in PaCd_2 ; $\bullet$, site 2 in PaCd_2 ; $\square$, site 1 in PaCdLu ; $\blacksquare$, site 2 in PaLaCd .

comes too low to be observable. The intensity of resonance 2' goes through an intensity maximum for $R = 1$. On the assumption that resonances 2 and 2' are due to the same site in PaCd_2 and PaCdLu respectively (where Pa represents parvalbumin), we find that La^{3+} has a slight preference for site 1.

The titration with Lu^{3+} also results in a simultaneous decrease in resonances 1 and 2, and a new resonance labelled 1' appears approx. 1 p.p.m. downfield from resonance 1. Resonances 1 and 1' are shifted upfield with increasing amounts of Lu^{3+} and are assigned to the same site, whereas resonance 2 does not shift (see Fig. 2). The intensity of resonance 1' increases up to $R = 1$ and stays almost constant for higher ratios (Fig. 1). Again on the assumption that resonances 1 and 1' are due to the same site in PaCd_2 and PaCdLu respectively, we conclude that Lu^{3+} has a strong preference for site 2 in PaCd_2 .

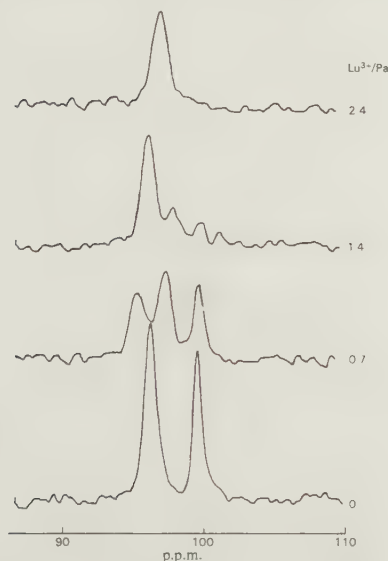


Fig. 2. ^{113}Cd -n.m.r. spectra of 2mM- Cd^{2+} -saturated carp (pI4.25) parvalbumin in 50mM-sodium cacodylate buffer, pH6.1, as a function of added Lu^{3+} .

The Lu^{3+} /parvalbumin ratios are indicated in the Figure.

The titration with Gd^{3+} results in large and selective broadening of resonance 1. The intensity of resonance 2 decreases faster than that of resonance 1. In earlier experiments performed at lower frequency and lower S/N ratios, the broadening of signal 1 was overlooked and as a result was erroneously interpreted as a decrease in intensity (Drakenberg *et al.*, 1978). The addition of large excess of Mg^{2+} (about 300equiv.) suppresses the broadening induced by 0.15equiv. of Gd^{3+} on resonance 1 and shifts this resonance to high field, as previously described for similar amounts of Mg^{2+} (Cavé *et al.*, 1979b, 1982), showing that Mg^{2+} and Gd^{3+} compete for the same binding site and that the ratio in the binding constants, $K^{\text{Cd}}/K^{\text{Mg}}$, can be estimated to be between 100 and 1000.

The titration with either Tb^{3+} or Yb^{3+} results in simultaneous decrease of resonances 1 and 2 and appearance of a new resonance labelled 1', which goes through an intensity maximum at about $R = 1$. The chemical shift of resonance 2 stays con-

stant throughout both titrations, whereas the shift of both resonances 1 and 1' depends on the amount of added lanthanide ions. For small amounts of lanthanide ions ($R < 0.5$) signal 1 shifts upfield, as for La^{3+} and Lu^{3+} , and continues to shift that way also for $R > 0.5$ for Yb^{3+} . For Tb^{3+} the shift variations on resonances 1 and 1' are reversed for $R > 1$. On the same assumption as above with regard to the assignments of the resonances we find that both Tb^{3+} and Yb^{3+} have a preference for site 2 in PaCd_2 . This preference is more pronounced in the case of Yb^{3+} , but, however, not as pronounced as for Lu^{3+} .

^{113}Cd -n.m.r. study of pike parvalbumin (pI5.0)

$^{113}\text{Cd}^{2+}$ -saturated pike pI5.0 parvalbumin was titrated with La^{3+} , Gd^{3+} , Yb^{3+} , Lu^{3+} and Mn^{2+} . Fig. 3 shows the variation in the ^{113}Cd resonances as a function of added La^{3+} or Lu^{3+} . Titration with La^{3+} results in very similar decreases in resonances 1 and 2. Two additional resonances, labelled 1' and 2', appear. The intensity of signal 1' is about twice that of signal 2'. The chemical shift of resonances 1 and 1' remain constant, whereas resonances 2 and 2' are shifted slightly to higher field with increasing La^{3+} concentration, and therefore signals 1 and 1' are assigned to one of the two strong sites in the protein and signals 2 and 2' are assigned to the other site. We thus find that La^{3+} has a slight preference for site 2 in PaCd_2 .

Titration with Lu^{3+} results first in a decrease in the intensity of resonance 1, which has almost disappeared at $R = 1$. For higher Lu^{3+} concentrations signal 2 starts to decrease in intensity and shifts to higher field. No extra signal due to Cd^{2+} bound to the protein appears during this titration (Fig. 4). This experiment shows that Lu^{3+} has a strong preference for site 1.

Titration with Gd^{3+} results in a large and selective broadening of resonance 2. The addition of 0.3equiv. of Gd^{3+} causes a broadening of 45 Hz, which is about half that observed for resonance 1 from carp parvalbumin under the same conditions. The addition of Gd^{3+} does not affect either the chemical shift or the line width of resonance 1, which is in agreement with previous observations for resonance 2 from carp parvalbumin (Drakenberg *et al.*, 1978). Similarly the intensity of resonance 1 from pike parvalbumin, like that of resonance 2 from carp parvalbumin, decreases faster than that of the other resonance, which is broadened by Gd^{3+} in both proteins.

Titration with either Tb^{3+} or Yb^{3+} leads to a faster decrease in the intensity of resonance 1 than in that of resonance 2. The chemical shift of resonance 1 does not vary in any of these titrations. Resonance 2, however, shifts by approx. 4p.p.m. to high field after the addition of 2equiv. of Yb^{3+}

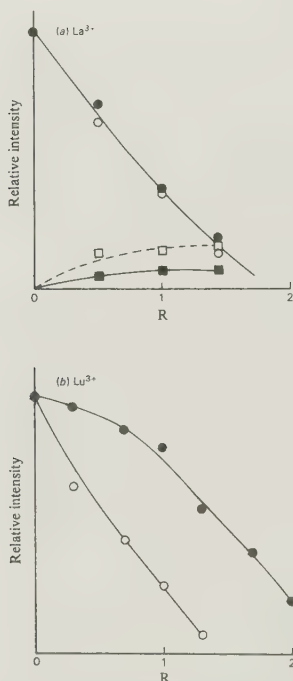


Fig. 3. Relative intensities of the ^{113}Cd -n.m.r. resonances from Cd^{2+} bound to pike (p15.0) parvalbumin as a function of added La^{3+} or Lu^{3+} .

The solution contained 2mM-PaCd₂ in 50mM-sodium cacodylate buffer, pH6.1. O, Site 1 in PaCd_2 ; ●, site 2 in PaCd_2 ; □, site 1 in PaCdLa ; ■, site 2 in PaLaCd .

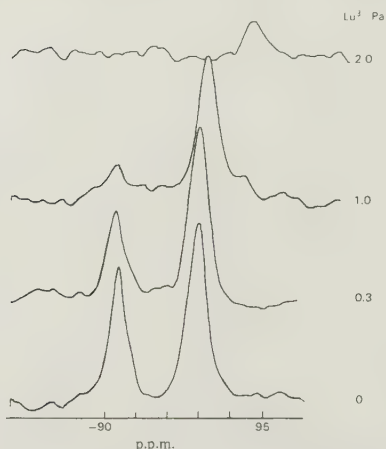


Fig. 4. ^{113}Cd -n.m.r. spectra of 2mM- Cd^{2+} -saturated pike (p15.0) parvalbumin in 50mM-sodium cacodylate buffer, pH6.1, as a function of added Lu^{3+} .

The $\text{Lu}^{3+}/\text{parvalbumin}$ ratios are indicated in the Figure.

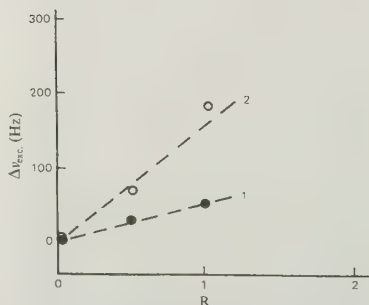


Fig. 5. Excess broadening of the ^{113}Cd -n.m.r. resonances from a 3mM solution of Cd^{2+} -saturated pike (p15.0) parvalbumin in 50mM-sodium cacodylate buffer, pH6.1, as a function of added Mn^{2+} . $R = \text{Mn}^{2+}/\text{Pa}$.

and by approx. 2p.p.m. to low field after the addition of 2equiv. of Tb^{3+} . No additional resonances could be observed in any of these two cases, in agreement with the Lu^{3+} competition but opposite to the results obtained with La^{3+} . Both Tb^{3+} and Yb^{3+} show a preference for site 1, Yb^{3+} more so than Tb^{3+} , though not as much as Lu^{3+} .

Titration with Mn^{2+} results in significant broadening of both resonances (Fig. 5). However, resonance 2 broadens twice as much as resonance 1. These broadenings are much less than what was previously observed for the carp protein (Cavé *et*

al., 1982). It may also be noted that the broadening induced by Mn^{2+} is much more nearly linearly dependent on R than is that induced by Gd^{3+} for both proteins.

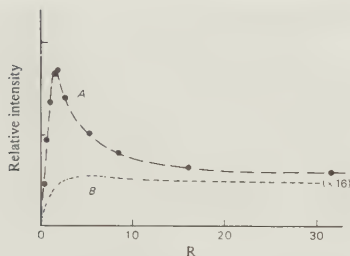


Fig. 6. Tb^{3+} -fluorescence study of Tb^{3+} binding to pike (pI 5.0) parvalbumin

The parvalbumin at 2.5 mM (curve A) and 0.01 mM (curve B) in 50 mM-piperazine buffer containing 0.1 M-KCl was titrated with $Tb(NO_3)_3$. The fluorescence intensity in arbitrary units is plotted versus $R = Tb^{3+}/Pa$.

Tb^{3+} fluorescence

The Tb^{3+} fluorescence emission at 545 nm that is observed when the aromatic chromophore of pike pI 5.0 parvalbumin is irradiated at 259 nm is shown in Fig. 6 as a function of Tb^{3+} concentration. The titration curve obtained for the highest concentration of protein (2.5 mM) is similar to that previously reported for the carp parvalbumin (Nelson *et al.*, 1977). Note that quenching of the fluorescence takes place at higher Tb^{3+} concentrations. At lower pike protein concentration (0.01 mM) a titration curve of different shape is observed, as depicted in Fig. 6(b). Here the fluorescence intensity does not decrease at higher Tb^{3+} concentrations. Under similar conditions the carp protein, however, still presents the quenching effect (results not shown) previously described (Nelson *et al.*, 1977).

Discussion

Assignment of the ^{113}Cd -n.m.r. resonances

In a preliminary ^{113}Cd -n.m.r. study on carp parvalbumin we made a tentative assignment of the two observed ^{113}Cd -n.m.r. resonances to the CD and EF sites (Drakenberg *et al.*, 1978). This assignment was, however, based on an erroneous interpretation of the Cd^{2+} - Gd^{3+} competition experiment, and has unfortunately also been used in a ^{113}Cd -n.m.r. study of a series of α -lineage and β -lineage parvalbumins (Cavé *et al.*, 1982). With the better data now at hand for the competition between Cd^{2+} and various lanthanide ions, especially Tb^{3+} , we wish to re-evaluate the assignments.

It has been shown that Tb^{3+} , at low concentrations, in crystals selectively replaces Ca^{2+} in the EF site before replacing the Ca^{2+} in the CD site in carp parvalbumin (Sowadski *et al.*, 1978). Furthermore, it has been shown that there is no selectivity in the Ca^{2+} - Cd^{2+} competition (Drakenberg *et al.*, 1978), which has led us to the conclusion that Tb^{3+} will show a preference for the EF site in $PaCd_2$ as well as in $PaCa_2$. We therefore make a tentative assignment of the high-field ^{113}Cd resonance ($\delta = -99.5$ p.p.m.) in carp parvalbumin to the EF site. We note that, even though this metal ion is thought to be accessible to solvent (Kretsinger, 1976), its ^{113}Cd -n.m.r. chemical shift does not depend on pH or metal-ion concentration, whereas the chemical shift of the other ^{113}Cd resonance depends strongly on excess metal ions. This assignment is therefore in agreement with the suggestion by Rhee *et al.* (1981) that the third metal-ion-binding site is situated close to the CD site and shares the carboxylic groups from Asp-53 and Glu-59 with the CD site. On the assumption that the CD-site ^{113}Cd resonance is the one that is sensitive to excess metal ions whether there is a Cd^{2+} ion or a lanthanide ion in the EF site, we can also assign resonance 2' from the La^{3+} competition to the EF site in $PaLaCd$ and resonance 1' from the Lu^{3+} competition to the CD site in $PaCdLu$.

There is no X-ray-crystallographic structure available for any α -lineage parvalbumin; however, there seems to be a general agreement that the structure is well conserved within the parvalbumin family of proteins. On the basis of this assumed structural similarity we may deduce that the observed variation in the chemical shift in one of the ^{113}Cd resonances from Cd^{2+} bound to pike parvalbumin as a function of added lanthanide ions is caused by ion binding to the third site, close to the CD site, as in the β -lineage parvalbumins. For the pike protein we have observed that it is the low-field ^{113}Cd resonance that is not affected by the addition of lanthanides and therefore tentatively assigned to the EF site. Similarly we can assign resonances 1' and 2' in the La^{3+} competition to the EF and CD sites respectively in the mixed complexes. This is the only example where we have observed all four possible ^{113}Cd -n.m.r. resonances, i.e. two from $PaCd_2$ and one each from $PaCd^{EF}La^{CD}$ and $PaLa^{EF}Cd^{CD}$.

Lanthanide-ion- Cd^{2+} competition

Figs. 1 and 3 show that, for both the β -lineage parvalbumin from carp and the α -lineage parvalbumin from pike, La^{3+} as well as Lu^{3+} will compete favourably with Cd^{2+} for at least one of the two primary sites. According to our assignments above, Lu^{3+} will bind preferentially to the EF site in carp parvalbumin and does not compete

very effectively with Cd^{2+} bound to the CD site, whereas La^{3+} shows a slight preference for the CD site. Lee & Sykes (1981) and Corson *et al.* (1983a) have found, by using proton n.m.r., that Yb^{3+} and Lu^{3+} bind preferentially to the EF site of the Ca^{2+} -saturated protein. However, they also observed a strong competition between these ions and Ca^{2+} for the CD site. This difference in the competition for the CD site is most probably due to the fact that in the present work we have studied the competition between lanthanide ions and Cd^{2+} rather than Ca^{2+} ions. Cd^{2+} ions are known to bind more strongly to parvalbumins than do Ca^{2+} ions (Cavé *et al.*, 1979a, 1981).

Tb^{3+} and Yb^{3+} show competition with Cd^{2+} similar to that shown by Lu^{3+} for the two primary sites. There is only a small variation in how effective they are in competing with the Cd^{2+} ions for the CD site. Tb^{3+} is the most and Lu^{3+} the least effective. This is in qualitative agreement with the data reported by Corson *et al.* (1983a). They found that $K_{\text{L}}^{3+}/K_{\text{C}}^{3+}$ is twice as large for the CD site as for the EF site, whereas $K_{\text{L}}^{3+}/K_{\text{C}}^{3+}$ is 8 times as high for the EF site as for the CD site. The whole variation lies in the CD site, whose relative binding constant increases 18-fold from Lu^{3+} to La^{3+} , resulting in a slight preference of La^{3+} for the CD site and a strong preference of Lu^{3+} for the EF site.

To our knowledge there is, before this present work, no information available on the lanthanide ion- Ca^{2+} or lanthanide ion- Cd^{2+} ion competition for a parvalbumin belonging to the α -phylogenetic lineage. Our results, however, show quite convincingly that the two proteins studied behave very similarly with regard to the metal-ion competition. La^{3+} shows a slight preference for the CD site, and Lu^{3+} prefers the EF site strongly. There is, however, a difference in how well Lu^{3+} competes with Cd^{2+} for the EF site, as can be seen from Figs. 2 and 4. The ratio $K_{\text{L}}^{3+}/K_{\text{C}}^{3+}$ is larger for the pike protein (≥ 1) than for the carp protein (< 1).

The third site

When the Ln^{3+} - Ca^{2+} competitive binding to parvalbumin is monitored by, for example, Tb^{3+} fluorescence, the change in the fluorescence is found to go through a maximum for less than 2 equiv. of Tb^{3+} per parvalbumin molecule, and decreases at higher Tb^{3+} concentration. This quenching has been explained as caused by binding of Tb^{3+} ions to a third site close to one of the primary sites (Rhee *et al.*, 1981). In X-ray-crystallographic studies there was no sign of such a site (Sowadski *et al.*, 1978). Also, in ^{113}Cd -n.m.r. studies effects due to metal-ion binding to a third site were observed (Cavé *et al.*, 1979b, 1982). However, these effects were observed only for the β -

phylogenetic-lineage proteins. In the latter studies it was observed that univalent cations caused a shift of the ^{113}Cd resonances from one of the sites to lower field, whereas bivalent cations caused a shift to higher field (Cavé *et al.*, 1982). The ^{113}Cd -n.m.r. signal from the same site is also broadened by the addition of paramagnetic ions such as Mn^{2+} . From this broadening the distance between the third site and a Cd^{2+} ion in the CD site was estimated to be less than 0.5 nm. Likewise, the distance between the third site and the EF site was estimated to be more than 1.0 nm (Cavé *et al.*, 1982).

Various effects are observed on the ^{113}Cd resonance from one of the sites of the carp pI4.25 protein when lanthanide ions are added to the Cd^{2+} -loaded protein: broadening with Gd^{3+} ions, upfield shift with the diamagnetic La^{3+} and Lu^{3+} , as well as with Yb^{3+} , and downfield shift with Tb^{3+} . The broadening induced by Gd^{3+} and the different chemical shifts caused by Tb^{3+} as compared with Yb^{3+} are related to the paramagnetic properties of these ions, which act as relaxation or shift probes on the neighbouring $^{113}\text{Cd}^{2+}$ ions when they are bound to the third, secondary, site. The initial shift effect induced by the addition of the various lanthanide ions is always the same, and is most probably caused by the binding of the released Cd^{2+} ions to the third site, since an addition of excess Cd^{2+} results in similar shifts.

In our earlier ^{113}Cd -n.m.r. studies (Cavé *et al.*, 1982) we used the fact that Mg^{2+} selectively causes a high-field shift for the ^{113}Cd resonance of the CD site to assign the resonances for several parvalbumins belonging to the β -phylogenetic lineage (note that the assignments have been reversed). However, the ^{113}Cd -n.m.r. spectra of parvalbumins belonging to the α -lineage were found to be insensitive to addition of Mg^{2+} or other bivalent or univalent cations, and no assignments could be made. We have now observed that the lanthanide ions will cause shift and/or line broadening of the ^{113}Cd resonance from one of the primary sites for the α -lineage parvalbumin from pike. Thus, in contrast with uni- and bi-valent cations, the trivalent lanthanide ions do affect the chemical shift of the high-field-shifted ^{113}Cd resonance of pike parvalbumin in a fashion similar to that observed for the low-field ^{113}Cd resonance of carp parvalbumin. On the basis of an inspection of the three-dimensional structure of the carp pI4.25 parvalbumin Rhee *et al.* (1981) proposed that the third binding site is situated close to the CD site. This suggestion is confirmed by our ^{113}Cd -n.m.r. results. The only difference between the α -lineage and β -lineage parvalbumins in the amino acid residues suggested to comprise the third site is the

substitution of Asp-61 in the β -lineage proteins for Glu-61 in α -lineage proteins.

The Tb^{3+} -fluorescence titration of the pike parvalbumin, as shown in Fig. 6, displays a quenching effect similar to the one observed for the carp parvalbumin. For the pike protein the quenching disappears at lower protein and Tb^{3+} concentrations. This was not observed in similar studies of the carp protein. This different behaviour for the two proteins is in agreement with the proposal of a weaker third site in the pike protein as compared with the carp protein. Curve A in Fig. 6 can be used to estimate the binding constant of Tb^{3+} to this site in the pike parvalbumin to be approx. 100 M^{-1} . The binding constant for the binding of this cation to the third site in the carp protein can be estimated to be $> 10^4\text{ M}^{-1}$.

We therefore propose that the α -lineage parvalbumins also have a secondary site, assumed to be close to the CD site. However, this site is much weaker in the α -lineage proteins than in the β -lineage proteins and apparently only binds tervalent cations to a noticeable amount under our experimental conditions. This would explain why this site is not observed as an Mg^{2+} site in ^{25}Mg -n.m.r. or ^{113}Cd -n.m.r. experiments (Cavé *et al.*, 1979b, 1982). Nevertheless, this weak third site may be useful to assign the ^{113}Cd resonances in the parvalbumins from the α -lineage.

Intersite interaction

We have already noted above that there is an interaction between the metal ion bound to the CD site and the one bound to the third site, evidenced in ^{113}Cd n.m.r. as well as in Tb^{3+} fluorescence. This interaction is probably relayed via one or two bridging carboxy groups and does not necessarily alter the structure of the protein. There is also in some examples a noticeable interaction between the two primary sites, which can be observed with ^{113}Cd n.m.r. As can be seen in Fig. 2, there are during the Lu^{3+} titration transiently more than two ^{113}Cd resonances from Cd^{2+} bound to the protein. The same is true for the La^{3+} competition experiments for both the carp protein and the pike protein. The new line(s) appearing is most easily explained as being due to the species PaCdLn (where Ln represents lanthanide). This therefore shows that the Cd^{2+} bound to one of the primary sites will sense whether there is a Cd^{2+} or a lanthanide ion in the other primary site. Since this effect is observed for the diamagnetic ions La^{3+} and Lu^{3+} , as well as for the paramagnetic lanthanide ions, we conclude that there is a, maybe slight, change in one of the Ca^{2+} -binding sites

when Ca^{2+} or Cd^{2+} in the other is replaced by a lanthanide ion. Similar and more dramatic intersite interactions have also been observed for the pair of Ca^{2+} binding sites in the intestinal Ca^{2+} -binding protein (Vogel *et al.*, 1985).

We are indebted to Professor B. D. Sykes for communicating results before publication and to Professor S. Forsén and Dr. H. J. Vogel for helpful discussions. This project was sponsored by grants from the Swedish Natural Sciences Research Council (N.F.R.) and the European Molecular Biology Organization (E.M.B.O.) (to A. C.).

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Cation binding to parvalbumin studied by ^{113}Cd and ^{23}Na NMR. Peak assignment of rabbit (pI 5.5) parvalbumin

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ABSTRACT

Cation binding to three apo parvalbumins has been studied by means of ^{113}Cd NMR. The three parvalbumins that have been investigated are carp pI 4.25, rabbit pI 5.5 and pike pI 5.0. The results show that Cd^{2+} ions bind to the EF and CD sites of carp apo parvalbumin pI 4.25 with about the same affinity. For rabbit (pI 5.5) apo parvalbumin the Cd^{2+} binds preferentially to the EF site while for pike (pI 5.0) apo parvalbumin it is the CD site that exhibits somewhat higher affinity for Cd^{2+} . The effect of Mn^{2+} on the ^{113}Cd signals of rabbit parvalbumin has been used to assign the ^{113}Cd NMR signals to the EF and CD sites. The Mn^{2+} paramagnetic effect on rabbit and pike parvalbumins differs from what has been obtained for carp parvalbumin. This is in agreement with the assumption that the β -lineage parvalbumins possess a third external site of higher affinity than for the α -lineage parvalbumins.

Furthermore, ^{23}Na NMR has been used to study $\text{Na}^+ - \text{Mg}^{2+}$ competition in the native carp (pI 4.25) parvalbumin. The results show that Na^+ and Mg^{2+} compete for the same site, the third external site.

INTRODUCTION

Parvalbumins constitute a family of Ca^{2+} -binding proteins found in rather high concentrations in the sarcoplasm of vertebrate skeletal muscles. The physiological function of parvalbumins is not fully understood but one suggestion is that parvalbumin acts as a buffer for Ca^{2+} in the relaxation process of the muscle.¹⁻³

The crystalline structure of one parvalbumin, component pI = 4.25 from carp, has been determined by X-ray diffraction.⁴

The X-ray crystallographic structure of carp parvalbumin shows that the polypeptide chain is arranged in six helical fragments, denoted A to F. The two calcium binding sites are formed in the loops between the C and D helices and E and F helices respectively and are therefore termed the CD and the EF sites. The ligands in the sites are oxygen atoms in carboxyl, carbonyl and hydroxyl groups which are octahedrally arranged around the metal ion.⁴

In the study of cation binding to calcium binding proteins, ^{113}Cd NMR has been shown to be of particular importance.⁵⁻⁹ The two calcium ions in the native protein can be readily replaced by $^{113}\text{Cd}^{2+}$ which possesses suitable magnetic properties for NMR studies. The two ions have about the same ionic radius ($r_{\text{Cd}^{2+}} = 0.97 \text{ \AA}$, $r_{\text{Ca}^{2+}} = 0.99 \text{ \AA}$) and both ions bind preferentially to oxygen atoms. ^1H NMR spectra of carp parvalbumin saturated with either Ca^{2+} or Cd^{2+} have been observed to be very similar.¹⁰

The chemical shift of the ^{113}Cd is very sensitive to the number and type of ligands and the chemical shift range extends over about 800 ppm.¹¹ The strong binding of Cd^{2+} ions to parvalbumins together with the very sensitive chemical shift of the nuclei makes a direct observation of $^{113}\text{Cd}^{2+}$ -ions bound to the CD and EF sites possible.

The parvalbumins have been classified, on the basis of their primary structure, into α - and β -parvalbumins.¹² Differences in cation binding properties between the α - and the β -proteins have been observed from ^{113}Cd NMR.^{5,6}

In this work, the binding of Cd^{2+} ions to three apo-parvalbumins has been studied using ^{113}Cd NMR. One of the proteins, carp pI 4.25, belongs to the β -lineage in the phylogenetic tree as described by Pechere.¹² The other two proteins, rabbit pI 5.5 and pike pI 5.0 belong to the α -lineage.

^{23}Na NMR has also been employed to study $\text{Na}^+ - \text{Mg}^{2+}$ binding and competition to the native carp (pI 4.25) parvalbumin.

Experimental

Rabbit parvalbumin was prepared, with slight modification, according to Leliky et al.¹³

Pike parvalbumin was prepared by Eva Thulin, Dept. of Physical Chemistry 2, Chemical Center, Lund, Sweden and carp parvalbumin was supplied by Professor J. Parello, Montpellier, France. Purity of the proteins was checked from agarose gel electrophoresis.

Apo-parvalbumin, that is Ca^{2+} -free protein was prepared by passing an aqueous solution of the protein through a column of Chelex-100. The lyophilized apo-protein was then dissolved in appropriate volumes of water or buffer solution at the time of use.

A 0.1 M ^{113}Cd -solution was prepared by dissolving CdO (96.3% isotopically enriched in ^{113}Cd , Oak Ridge, National Laboratory, Oak Ridge, TN) in HClO_4 . Adjustment of pH was made with Tris to a final pH value of 6.4. Final concentration of HClO_4 and Tris was 0.25 M and 0.125 M respectively.

Protein concentrations were determined either by weighing or by UV-absorption measurements according to Cavé et al.¹⁴

All NMR spectra were obtained on a home-made Fourier transform spectrometer equipped with an Oxford Instruments 6T wide-bore magnet. The ^{113}Cd NMR chemical shifts are given relative to the shift of an aqueous 0.1 M Cd (ClO_4)₂ solution with high-field shifts given negative values.

Results and Discussion

The effect of Mn^{2+} on the ^{113}Cd NMR signals of rabbit (pI 5.5) parvalbumin. Figure 1 shows the Mn^{2+} linebroadening effect on the ^{113}Cd NMR signals of rabbit (pI 5.5) parvalbumin. One of the signals is slightly more affected than the other one. In agreement with other parvalbumins belonging to the α -lineage in the phylogenetic tree.⁷ This result has been used to assign the ^{113}Cd NMR signals of rabbit to the CD and EF sites.

A dramatic effect of the paramagnetic ion Mn^{2+} on the ^{113}Cd spectra from the Cd^{2+} loaded carp pI 4.25 parvalbumin has been observed by Cavé et al.⁶, only one of the ^{113}Cd NMR signals showing linewidth dependence upon Mn^{2+} concentration. This effect was attributed to the binding of Mn^{2+} to the third external site close to the CD high-affinity binding site. The effect of Mn^{2+} on $^{113}\text{Cd}^{2+}$ loaded pike (pI 5.0) parvalbumin was somewhat different.⁶ For the pike protein both ^{113}Cd signals were broadened in a similar way as for the rabbit (pI 5.5) parvalbumin.

Drakenberg et al.^{6,7} showed, from lanthanide- $^{113}\text{Cd}^{2+}$ competition experiments on pike that there exists a third external site also in this α -lineage protein. However, for the pike protein it is weaker than for the β -lineage protein of carp (pI 4.25). Based on these results we can thus

conclude that the signal that is slightly more broadened upon addition of Mn^{2+} on rabbit (pI 5.5) α -lineage parvalbumin corresponds to the CD-site. This means that the low-field shifted ^{113}Cd NMR signal of rabbit corresponds to the CD site. Consequently, the ^{113}Cd NMR peak assignments of rabbit (α) and carp (β) are the same, while for pike it is the reverse.

Addition of $^{113}Cd^{2+}$ to apo-parvalbumins. Figure 2 shows the spectral appearance as $^{113}Cd^{2+}$ ions are added to the carp pI 4.25 apo-protein. The two signals that correspond to the EF- and CD-site bound Cd^{2+} ions rise simultaneously. The high-field signal i.e. EF(Cd^{2+}), shows only slightly higher affinity for Cd^{2+} ions. The assignment of the signals to the EF- and CD-sites are according to Drakenberg et al.⁷ At low $[Cd^{2+}]$ to $[protein]$ ratio (0.5) it is possible to distinguish an additional slightly shifted signal, of less intensity, to each of the two higher intensity signals. These signals of lower intensity probably arise from protein molecules where only one of the sites is occupied by Cd^{2+} . It has been observed, that the apo-protein is somewhat less structured than the Ca^{2+} -loaded protein but that the structure is restored as Ca^{2+} ions are bound.¹⁵

Figure 3 shows ^{113}Cd spectra for pike pI 5.0 at different $[Cd^{2+}]$ to $[protein]$ ratios. The peak assignments for the fully $^{113}Cd^{2+}$ loaded pike parvalbumin is the reverse of that of carp pI 4.25. The two signals around -90 ppm are probably due to Cd^{2+} ions in the EF site. The intensity of the signal at -90.5 ppm decreases as Cd^{2+} content increases and is therefore likely to correspond to EF- Cd^{2+} when there is no Cd^{2+} in the CD-site. The signal at -89.5 ppm corresponds to EF- Cd^{2+} when the CD-site is filled.

Figure 4 shows the ^{113}Cd spectral appearances as Cd^{2+} ions are added to the apo-parvalbumin of rabbit (pI 5.0). The Cd^{2+} ions show a slight

preference for the EF-site. The peak assignments to the EF- and CD-sites are deduced from the effect of the paramagnetic ion Mn^{2+} . See discussion above and Figure 1. At $[\text{Cd}^{2+}]/[\text{protein}] = 0.45$ four signals appear, which are from the left to the right, likely to correspond to CD-Cd^{2+} (EF-0), CD-Cd^{2+} (EF- Cd^{2+}), EF-Cd^{2+} (CD-Cd^{2+}) and EF-Cd^{2+} (CD-0). The signal at -90 ppm has disappeared at $\text{Cd}^{2+} / \text{protein} = 0.9$ depicting the somewhat higher affinity for Cd^{2+} at the EF-site. The signals that correspond to the CD-site (-84.5 and -85.5 ppm) show the same behavior as the EF-site signals for the pike (pI 5.0) parvalbumin.

However, time dependent spectral appearances for pike parvalbumin (pI 5.0) with $[\text{Cd}^{2+}]/[\text{protein}] \approx 1$ has been observed by Drakenberg (unpublished results). This time dependence implies that the apo-protein regains a more structured conformation quite slowly as Cd^{2+} is added. This suggests that the ^{113}Cd chemical shifts, observed in this work may, change somewhat with time. It is clear, though, from lanthanide competition experiments with ^{113}Cd NMR that intersite interactions exist.⁷

$\text{Na}^+ - \text{Mg}^{2+}$ competition observed from ^{23}Na NMR. Figure 5 shows the excess ^{23}Na linewidth for a solution containing calcium loaded carp (pI 4.25) parvalbumin as a function of Mg^{2+} concentration. The observed line broadening effect on the ^{23}Na NMR signal in the protein solution shows that Na^+ binds to the protein. Furthermore, the decrease in linewidth upon Mg^{2+} concentration also shows that Na^+ and Mg^{2+} compete for the same site. It has been observed from ^{113}Cd NMR that Mg^{2+} does not compete with Cd^{2+} for the high-affinity sites.⁶ It can thus be concluded that Mg^{2+} and Na^+ compete for the third external site.

Earlier reports suggest that Na^+ binds with comparatively strong affinity for one of the high-affinity sites in a half-decalcified parvalbumin.^{16,17} In another report, no significant binding of Na^+ to parv-

albumin was observed and the results of Ref. 16 and 17 were attributed to an Na^+ -EGTA complex interacting with the protein.¹⁸ The result in Figure 5 shows that Na^+ binds to parvalbumin which contains Ca^{2+} in the two high-affinity sites and that Mg^{2+} compete with Na^+ for this site.

Concluding remarks

Important differences in ion binding properties between α - and β -lineage proteins have been demonstrated.^{6,7} From the work presented here it is clear that the differences still prevail even though the proteins have been deionized and in this form they are presumably in a less structured state.

In the carp parvalbumin (pI 4.25) the EF and CD sites bind Cd^{2+} with about the same affinity. In the pike (pI 5.0) and the rabbit (pI 5.5) parvalbumins, one of the sites binds Cd^{2+} slightly stronger.

For the pike parvalbumin the CD site is preferentially filled while for the rabbit parvalbumin it is the EF site that is filled first. Both these proteins belong to the α -lineage in the phylogenetic tree based on their primary structure.

The results presented here are in agreement with La^{3+} competition experiments with ^{113}Cd NMR.⁷ For the carp parvalbumin La^{3+} competes with Cd^{2+} in both the CD and the EF sites. When Cd^{2+} is added to the apo-protein, the CD and EF sites are filled simultaneously. The addition of La^{3+} to ^{113}Cd -loaded pike parvalbumin shows that La^{3+} goes preferentially into the CD site. The addition of ^{113}Cd to the apo-pike shows that the CD site is preferentially filled.

The linebroadening effect of the paramagnetic ion Mn^{2+} on ^{113}Cd NMR signals of rabbit (pI 5.5) parvalbumin has been used to assign the signals to the EF- and CD-sites. The rabbit parvalbumin which belongs to the

α -lineage in the phylogenetic tree has the same peak assignment of the β -lineage protein carp (pI 4.25).

Furthermore, results from ^{23}Na NMR show that Na^+ and Mg^{2+} compete for the same site, the third external site, in a Ca^{2+} -loaded carp (pI 4.25) parvalbumin.

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Figure legends

- Figure 1 Excess broadening of the ^{113}Cd NMR signals from 2.1 mM rabbit (pI 5.5) parvalbumin as a function of Mn^{2+} concentration. The signal which is somewhat more broadened than the other has been assigned to the CD-site bound $^{113}\text{Cd}^{2+}$. This signal is the low-field signal in the spectrum.
- Figure 2 ^{113}Cd NMR for carp (pI 4.25) parvalbumin at two different Cd^{2+} to protein ratios. The assignments of the signals are according to Drakenberg et al.⁷ This means that the low-field signal in the spectrum corresponds to the CD-site bound $^{113}\text{Cd}^{2+}$.
- Figure 3 ^{113}Cd NMR spectra for pike pI 5.0 parvalbumin at three different Cd^{2+} to protein ratios. The assignments of the signals are according to Drakenberg et al.⁷ Thus, the high-field signal corresponds to CD-site bound $^{113}\text{Cd}^{2+}$.
- Figure 4 ^{113}Cd NMR spectra for rabbit (pI 5.5) parvalbumin at three different Cd^{2+} to protein ratios. The peak assignments are based upon results from Mn^{2+} -linebroadening effects in Figure 1. Consequently, the signal at -89 ppm corresponds to the EF-site bound Cd^{2+} .
- Figure 5 Excess ^{23}Na linewidth for a 5 mM Na^+ solution, containing 3.8 mM carp (pI 4.25) parvalbumin as a function of Mg^{2+} concentration.

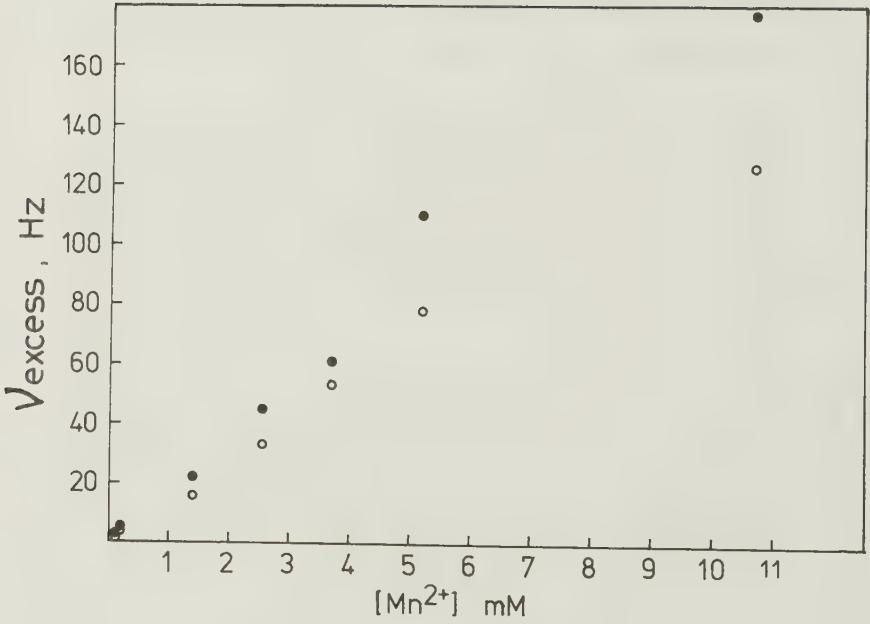


FIGURE 1

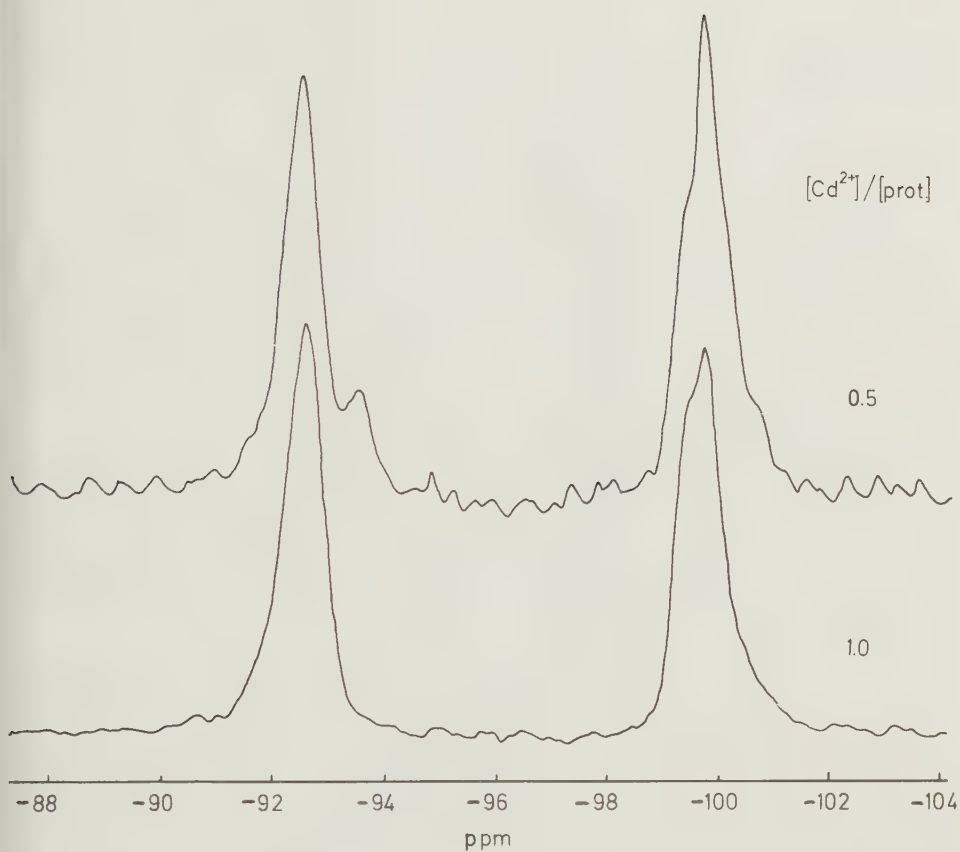


FIGURE 2

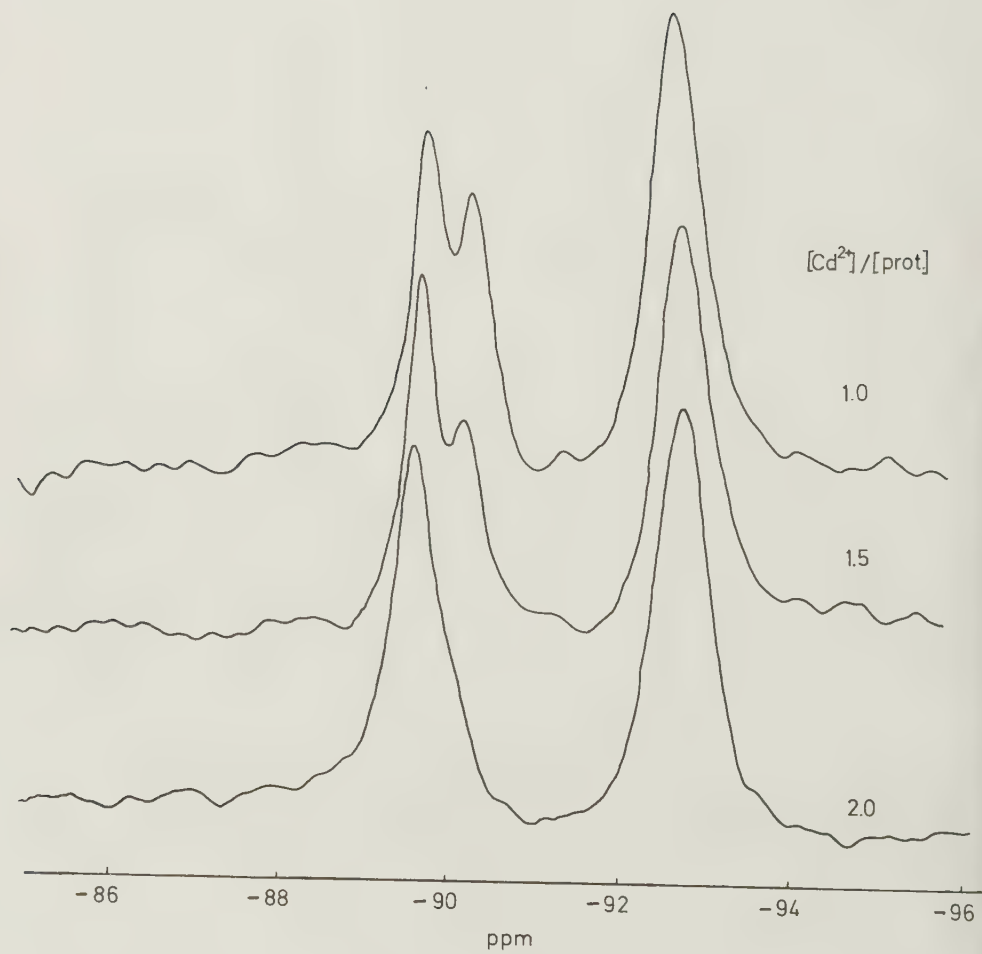


FIGURE 3

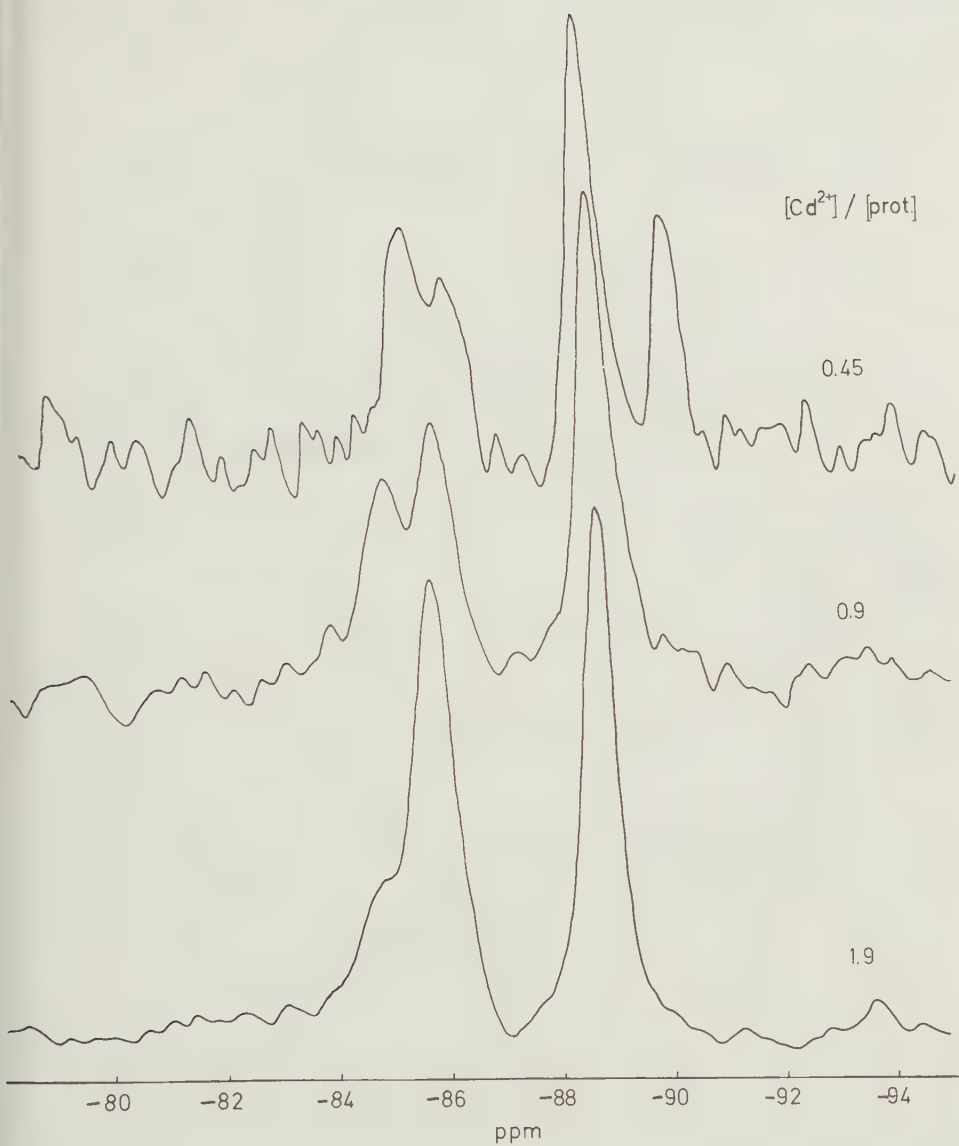


FIGURE 4

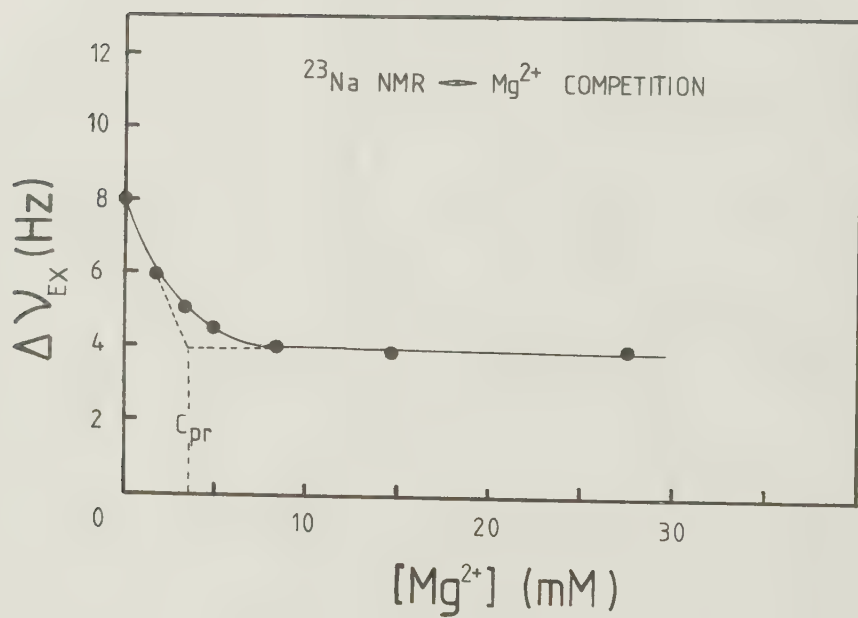


FIGURE 5

ARTICLE IV

CALCIUM BINDING TO BONE γ -CARBOXYGLUTAMIC ACID PROTEIN FROM CALF STUDIED

BY ^{43}Ca NMR

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NOTES:

1. Abbreviations used:

Gla, γ -carboxyglutamic acid

BGP, Bone Gla Protein, osteocalcin

HPLC, high performance liquid chromatography

SDS, sodium dodecyl sulphate

NMR, nuclear magnetic resonance

FOOTNOTES

The rotational correlation time (τ_c^{rot}) of BGP can be estimated to ~ 2 ns at 20°C from the Debye-Stokes-Einstein equation [24]. We are thus able to determine an upper limit of $\omega\tau_c$ in our experiments: $\omega\tau_c < 0.22$ ($\omega = 1.078 \cdot 10^8 \text{ s}^{-1}$ and $\tau_c < 2 \cdot 10^{-9} \text{ s}$).

ABSTRACT

Calcium binding to bone γ -carboxyglutamic acid protein (BGP) from calf has been studied using ^{43}Ca NMR. The temperature dependence of the ^{43}Ca NMR signal has been used to calculate the calcium ion exchange rate, k_{off} . The dependence of the ^{43}Ca NMR band shape on the $[\text{Ca}^{2+}]/[\text{BGP}]$ ratio fits well to a chemical equilibrium model having a single Ca^{2+} binding site with an association constant in the range of $5 \cdot 10^3 \text{ M}^{-1}$ to $1 \cdot 10^5 \text{ M}^{-1}$. The pH dependence of the ^{43}Ca NMR line width shows a single apparent pK_a value of 5.1.

INTRODUCTION

Bone from mammals, birds and fishes contains a low molecular weight non-collagenous protein, in which the unusual amino acid γ -carboxyglutamic acid is found [1,2]. The protein, which has been named osteocalcin or bone γ -carboxyglutamic acid protein (bone gla protein, BGP¹), consists of a single polypeptide chain of about 50 amino acid residues, two or three of which are γ -carboxyglutamic acid [3-5].

γ -Carboxyglutamic acid, which is also found in some of the coagulation factors and related plasma proteins [6], is formed by a vitamin K-dependent posttranslational carboxylation of certain glutamic acid residues in the protein polypeptide [7,8].

Most of the BGP is located in the extracellular bone matrix, adsorbed to the mineral crystals [9-12], but BGP also occurs in plasma at low concentrations [13-15]. Osteosarcoma cells with characteristics of osteoblasts have been shown to synthesize BGP [16], and the synthesis is stimulated by 1,25-dihydroxicholecalciferol [17].

BGP is a calcium binding protein [19-20] and the biological function of it may be assumed to be in some way related to this property. The detailed function of BGP is still unknown but long-term vitamin K deficiency in rats, which inhibits the formation of normal BGP, results in an over-mineralization of bone. BGP may therefore play a role in preventing excessive mineralization [18].

The calcium binding properties of BGP has been studied by various techniques [19-22] showing that there is at least one calcium binding site with a binding constant $K_B > 10^4 \text{ M}^{-1}$, and some weaker sites $K_B \approx 10^3 \text{ M}^{-1}$. It is naturally of interest to characterize the binding of calcium to BGP more closely and we have chosen to use the ^{43}Ca NMR

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technique which has proven to be very useful in the study of various kinds of calcium binding protein. BGP from calf bone was chosen because it is well characterized [3] and calf bone can easily be obtained in large quantities. A simple procedure for the large scale purification of BGP from calf bone is also presented.

MATERIALS AND METHODS

Preparation and characterization of BGP. BGP was isolated from calf bone by a large scale purification procedure. The isolated protein was characterized by SDS polyacrylamide gel electrophoresis, HPLC, amino acid analysis and sequence determination. The isolated BGP was judged to be at least 95% pure and the analytical data, including Gla content, were in good agreement with published data on the protein [2,3]. The details of the purification procedure and the results of the characterization of the purified protein are given in the Miniprint Supplement.

BGP solutions for NMR measurements. Ca^{2+} -free BGP was prepared by passing an aqueous solution of the protein through a column of Chelex-100 (BioRad Labs., Richmond, CA., USA) resulting in a protein containing less than 0.1 eqs. of Ca^{2+} . The solution was then lyophilized, and at the time of use dissolved in doubly distilled water. The pH of the solution was adjusted with 1M HCl or 1M NaOH. The concentration of BGP in the solution was determined spectrophotometrically, using the absorbance coefficient $A_{1\text{cm}}^{1\%} = 19.6$ at 280 nm (see the Miniprint Supplement).

Ca^{2+} -solutions for NMR measurements. A stock solution of CaCl_2 (115 mM) was prepared by dissolving CaCO_3 (60% isotopically enriched in ^{43}Ca , Oak Ridge Natn. Lab., Oak Ridge, TN., USA) in 1M HCl. The solution was neutralized to a final pH of 7.0 by adding 1M NaOH.

NMR measurements. The ^{43}Ca NMR spectra were recorded at 17.16 MHz on a home-built Fourier transform spectrometer as described elsewhere [23]. Depending on the Ca^{2+} concentration, 10^5 - 10^6 transients were accumulated using a 90° pulse ($\sim 50 \mu\text{s}$ pulse length). In order to have a negligible effect from probe ringing, a delay of $300 \mu\text{s}$ was introduced between the end of the pulse and the beginning of data accumulation.

Band shape calculations. The band shapes of the ^{43}Ca NMR signals from a solution where the Ca^{2+} ion exchanges between a protein site and free (solvated) Ca^{2+} ions depend on the exchange rate k_{ex} , the association constant K_a , and the relaxation rate of the ions bound to the protein, R_2 . It has previously been shown how to extract these parameters from the temperature and calcium concentration dependencies of the band shape of the ^{43}Ca NMR signal [23]. This method has been used here with the modification that now 17 curves at various temperatures and calcium concentrations have been used in one single calculation, instead of two separate calculations for the temperature dependence and the calcium concentration dependence. The shape of the ^{43}Ca NMR signals has been characterized by the width of the signal at 10 different heights. These observed data points (ca 170) are then compared with the calculated ones in an iterative way in order to obtain the best agreement between the observed and calculated band shapes. The error square sum (ESS) is used as a measure of the fit.

In all calculations reported here, the entropy of activation, $\Delta S^\ddagger$, for the Ca^{2+} exchange was assumed to be 0. Non-zero values did not improve the overall agreement between the observed and calculated band shapes. This means that $k_{\text{off}} = (kT/h)\exp(-\Delta H^\ddagger/RT)$.

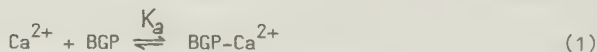
To give a visual representation of the result of the fitting procedure the line widths at half height for the calculated spectra are shown as solid lines in the figures. For the exchange broadened resonance, applicable

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to the present work, the width at half height does not represent a true relaxation rate, but can nevertheless be used to visualize the agreement between experimental and calculated line widths.

RESULTS

When BGP is added to a CaCl_2 solution at neutral pH the ^{43}Ca NMR line width is considerably broadened. Figure 1 shows the dependence of ^{43}Ca NMR line width on the Ca^{2+} concentration at a constant BGP concentration (1.85 mM). Figure 2 shows the temperature dependence of the ^{43}Ca line width for the same solution at a Ca^{2+} concentration of 10.7 mM. The data in Figure 1 and 2 were fitted to various models of chemical equilibria and it turned out that a simple model with one binding site fitted the data well.



With the Ca^{2+} and BGP concentrations used in the present work it is not possible to determine association constants greater than $5 \cdot 10^3 \text{ M}^{-1}$. We are thus only able to state that the association constant is greater than $5 \cdot 10^3 \text{ M}^{-1}$ if there is only one binding site. The exchange rate of Ca^{2+} ions was calculated to be $4.0 \pm 0.5 \cdot 10^3 \text{ s}^{-1}$.

It should be stressed that more complex models, i.e. multisite models also fit the data. Consequently, we cannot from these measurements rule out the presence of several equally strong Ca^{2+} binding sites or one strong and some weaker sites on BGP.

The pH dependence of the ^{43}Ca NMR line width for a 5.6 mM Ca^{2+} solution containing 1.4 mM BGP is shown in Figure 3. The decrease in the line width at low pH values is most likely due to protonation of the

Ca^{2+} binding carboxylate groups. The data can be fitted to the model of a single protonation step:

$$\Delta\nu = \Delta\nu_i / (1 + 10^{(\text{pK}_a - \text{pH})}) + C \quad (2)$$

In this equation $\Delta\nu_i$ is the increase in the line width associated with the apparent pK_a value. C is the line width that stays unaffected during the titration. When fitting Equation 2 to the data in Figure 3 we obtained the following parameters: $\Delta\nu_i = 61 \text{ Hz}$ and $\text{pK}_a = 5.1$. The constant term, C , was set equal to the line width of a pure Ca^{2+} solution, i.e., 14 Hz.

DISCUSSION

The calcium binding properties of BGP has been known for several years [19,20]. Equilibrium dialysis measurements suggest the presence of several sites on BGP from chicken bone [20]. The Scatchard analysis of the data indicated the presence of four Ca^{2+} binding sites; two "strong" sites ($K = 4 \cdot 10^4 \text{ M}^{-1}$) and two sites with lower affinity ($K = 1 \cdot 10^3 \text{ M}^{-1}$). Addition of cations such as Mg^{2+} and Na^+ indicated that these ions compete for the same sites [20]. Another equilibrium dialysis study indicates the presence of 1-2 Ca^{2+} binding sites with an association constant of the order of $3 \cdot 10^3 \text{ M}^{-1}$ [21]. The binding of Ca^{2+} to bovine BGP has also been studied by a gel filtration technique [19]. The data show however a considerable scatter. Circular dichroism measurements on chicken- [22] and bovine BGP [21] show that BGP undergoes a substantial conformational change upon binding of Ca^{2+} . The measurements indicate that there is an increase in the α -helical content from 5-10% to about 30-40%. This effect is also observed for other divalent and trivalent ions [21, 22].

The results of the ^{43}Ca temperature and Ca^{2+} -concentration dependence does not provide further details about the number of Ca^{2+} sites and the association constants to these sites. If it is assumed that there is one Ca^{2+} binding site the association constant is estimated to be greater than $5 \cdot 10^3 \text{ M}^{-1}$ and the exchange rate k_{off} , for Ca^{2+} is determined to $4.0 \pm 0.5 \cdot 10^3 \text{ s}^{-1}$.

An upper limit for the association constant can, however, be determined from the relation:

$$K_a = \frac{k_{\text{on}}}{k_{\text{off}}} \quad (3)$$

The value of k_{on} can not be greater than the exchange rate of the H_2O molecules surrounding in the first hydration shell at the Ca^{2+} ion, i.e.

$k_{\text{on}} < 5 \cdot 10^8 \text{ s}^{-1} \text{ M}^{-1}$ [25]. By using this value, equation 3 results in $K_a < 1 \cdot 10^5 \text{ M}^{-1}$. The Glu residues have been shown to participate in the Ca^{2+} binding [26]. The value of the association constant for Ca^{2+} to a single Glu residue (i.e. malonic acid) is $3 \cdot 10^2 \text{ M}^{-1}$ [27].

It is therefore not very likely that the Ca^{2+} binding site observed in this work is a surface site formed of a single Glu residue. There are two possibilities, either there is a surface site with more than one Glu residue participating in the Ca^{2+} -binding or there is a more buried site like in e.g. trypsin [28] or phospholipase [29]. Our data can not be used to a definite differentiation between these two possibilities.

However, the former is the more likely one because the calcium on-rate to the binding site appear to be limited by the water exchange rate, which it is not found to be in trypsin [28]. Also the presumed function of the protein as a regulator of bone mineralization points towards a surface site.

The pH dependence of the ^{43}Ca NMR line width shows a single apparent pK_a value of 5.1 and is about 0.6 pH units smaller than that reported by Hauschka and Gallop [20] for chicken BGP.

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FIGURE LEGENDS

Figure 1 The dependence of the ^{43}Ca NMR line width on the $[\text{Ca}^{2+}]/[\text{BGP}]$ ratio for a 1.85 mM BGP solution, pH = 7.2. The data were recorded at 21°C. The broken line shows the line width of Ca^{2+} solution without protein. The solid curve represents the result of the fitting procedure (see text).

Figure 2 The temperature dependence of the ^{43}Ca NMR line width for a 10.7 mM CaCl_2 solution, pH 7.2, containing 1.85 mM BGP. The broken line shows the line width of Ca^{2+} solution without protein. The solid curve represents the result of the fitting procedure (see text).

Figure 3 The pH dependence of the ^{43}Ca NMR line width for a 5.6 mM CaCl_2 solution containing 1.4 mM BGP. The data were recorded at 21°C. The solid line represents a fit of Equation 2 to the data; $\Delta\nu_i = 61$ Hz, $\text{pK}_a = 5.1$ and $C = 14$ Hz (i.e. the line width of the solution in the absence of protein, which is represented by the broken line).

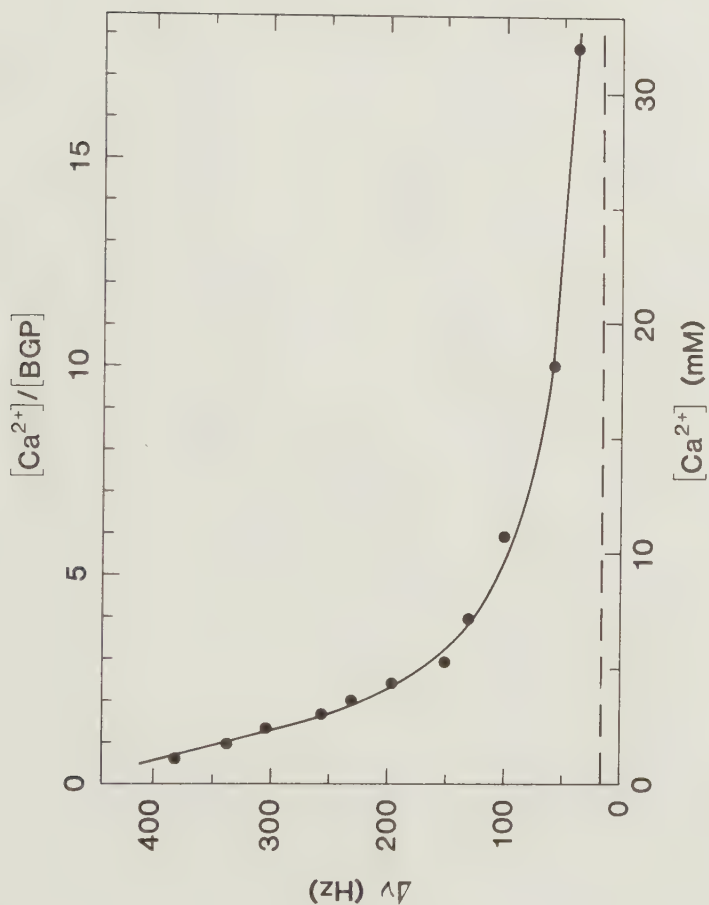


FIGURE 1

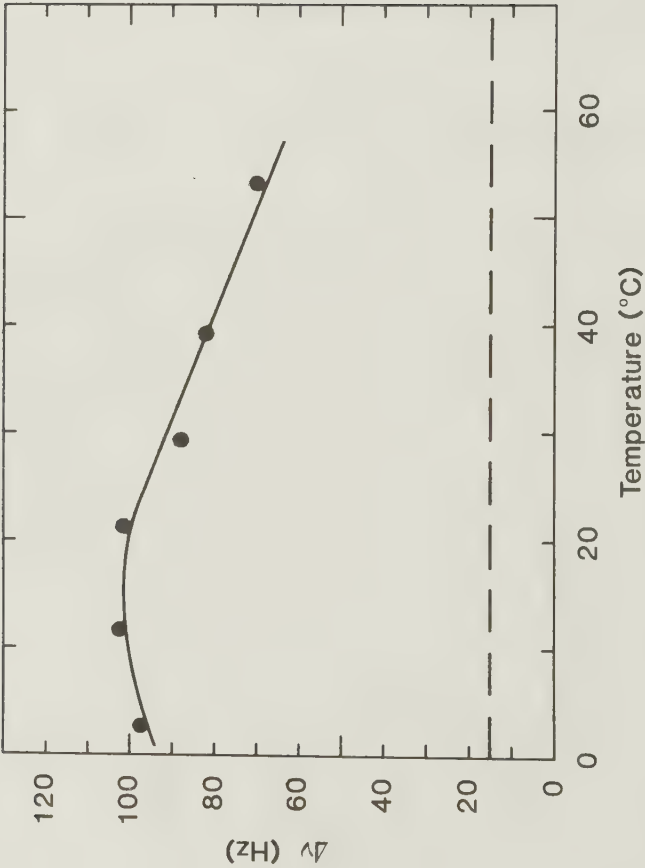


FIGURE 2

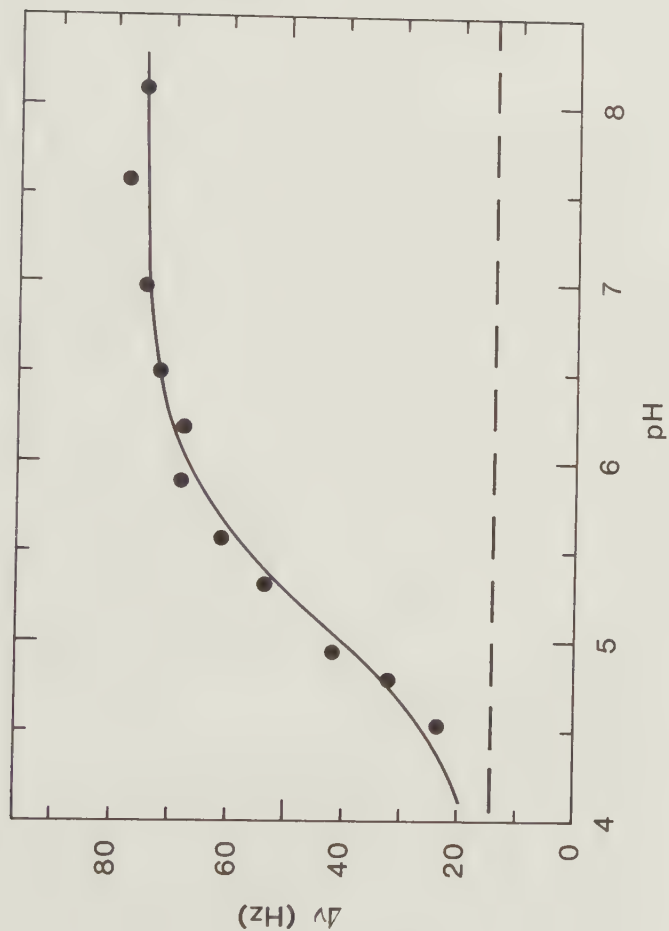


FIGURE 3

Supplementary Material to

Calcium Binding to Bone γ -Carboxyglutamic Acid Protein from Calf

Studied by ^{43}Ca -NMR

by

Marianne Svärd, Torbjörn Drakenberg, Thomas Andersson and Per Fernlund.

MATERIALS AND METHODS

Sephadex G-75, Superfine bead size, was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE 52) was obtained from Whatman Ltd., Maidstone, England. Spectrapore membrane tubing no. 3 (molecular weight cut off 3.5 kDa) was obtained from Spectrum Medical Industries Inc., Los Angeles, CA, USA. Phenylthiohydantoin (PTH) amino acids were purchased from Sigma Chemical Co., Saint Louis, MO, USA. DL- γ -carboxyglutamic acid was synthesized as described previously [30]. Its PTH-derivative was synthesized according to the procedure for PTH-serine as described by Edman [31]. All reagents used in automatic sequencing except for polybrene, which was obtained from Aldrich-Europe, Beerse, Belgium, were purchased from Beckman Instruments International, Geneva, Switzerland.

Agarose gel electrophoresis - Agarose slab gel electrophoresis was run in 1 mm thick, 1% agarose gels in 75 mM barbital buffer pH 8.6, 2.0 mM in calcium lactate or EDTA [32].

Amino acid analysis - BGP was hydrolysed in 6 M HCl at 110 °C in evacuated pyrex tubes with norleucine added as an internal standard. The hydrolysates were analysed in a Beckman 119 CL automatic amino acid analyser. Hydrolysis time was 24 h and no corrections were made for destruction during hydrolysis or

incomplete hydrolysis. γ -Carboxyglutamic acid was determined after alkaline hydrolysis as described previously [30].

Sequence determinations - Automatic Edman degradation [33] was performed with a Beckman Sequencer, model 890C. BGP was mixed with 3 mg of polybrene [34,35] and degraded with the standard Beckman no. 122974 Quadrol program. Thiazolinon amino acids were converted to PTH-amino acids and separated into ethylacetate and water phases by the standard procedure [32]. PTH-amino acids in ethylacetate and water phases, which were analysed separately, were identified by high performance liquid chromatography (HPLC) on a μ -Bondapak C₁₈ column (0.39 cm x 30 cm, Waters Associates, Inc., Milford, MO, USA) using stepwise elution with a system of methanol containing buffers [36].

PURIFICATION OF BGP

Calf tibias were obtained at slaughter. They were scraped free of adhering tissue, ground in a jaw crusher to a particle size of less than 3 mm and then kept at -20°C until extraction.

Extraction - 3 kg of ground bone tissue was stirred with 6 l of 30% (v/v) acetic acid at 4 °C for 24 h and the extract obtained after filtration of the mixture through ordinary filter paper was discarded. The remaining material was stirred for 6 days at 4 °C with another 6 l of 30% acetic acid. This extract, which was separated from insoluble material by centrifugation (6000 x g for 30 min, 4 °C), was dialysed in Spectrapore membrane tubing no. 3 against several changes of distilled water and then lyophilized.

DEAE-cellulose chromatography in calcium-free buffer - The lyophilized material (24.8 g) was stirred for 30 min at 4 °C

with 1000 ml of 50 mM Tris-HCl buffer pH 8.0, and the pH, which tended to decrease, was kept at 8.0 by the addition of 1 M NaOH. A small undissolved residue was discarded after centrifugation ($6000 \times g$ for 30 min, 4°C) and the supernatant was subjected to chromatography on a DEAE-cellulose column as described in the legend to Fig. 1. The Gla-containing fractions (A in Fig. 1) were pooled, dialysed against water and then lyophilized.

Sephadex G-75 chromatography - The material obtained in the previous step (620 mg) was dissolved in 35 ml of 50 mM Tris-HCl buffer pH 8.0, and subjected to chromatography on a column of Sephadex G-75 as described in the legend to Fig. 2. Fractions were pooled according to results of analysis of the effluent by agarose gel electrophoresis, dialysed against distilled water and then lyophilized.

DEAE-cellulose chromatography in calcium-containing buffer - The material obtained in the previous step was dissolved in 20 ml of 50 mM Tris-HCl buffer pH 8.0, and subjected to chromatography on a DEAE-cellulose column as described in the legend to Fig. 3. The effluent corresponding to the second of the two peaks in the chromatogram (A in Fig. 3) was combined, dialysed against water and then lyophilized yielding 47 mg of purified BGP.

CHARACTERIZATION OF PURIFIED BGP

The purification procedure is summarized in Table I. The yield of BGP from the extract used for the purification was 16% when estimated from the yield of Gla. This figure, however, represents a minimum since bone tissue contains Gla-containing proteins other than BGP [37]. A substantial amount of Gla-containing material was discarded with the first extract, from which purification of BGP was difficult.

Some Gla-containing material also remained in the insoluble residue.

The purified BGP was homogeneous according to analysis by agarose gel electrophoresis (not shown), sodium dodecyl sulfate, polyacrylamide gel electrophoresis (not shown), and HPLC (Fig. 4). The absorbance coefficient at 280 nm was 19.6 cm^{-1} for a 1% solution. The amino acid composition including the Gla-content (Table II) was in good agreement with the composition calculated from the sequence for bovine BGP given by Price et al [3]. Sequenator analysis also showed that the purified BGP was homogeneous and the H_2N -terminal sequence obtained (Table III) was identical to the sequence published [3]. Gla residues were positively identified in positions 17, 21 and 24.

Fig. 1. DEAE-cellulose chromatography of calf bone extract.

Material (dry weight 24.8 g) extracted from 3 kg of bone was dissolved in 1000 ml of 50 mM Tris-HCl buffer pH 8.0, (see the text), and pumped through a column (5 cm x 40 cm) of DEAE-cellulose. The column was then washed with 2 l of 50 mM Tris-HCl buffer pH 8.0, and finally eluted with a linear gradient of NaCl (0 to 1 M in 4 l) in the Tris buffer. The experiment was performed at 4 °C, the column had been equilibrated with the Tris buffer before the experiment and the flow rate was 56 ml/h through the whole experiment. Fractions of 16 ml were collected from the beginning of the elution and their absorbance at 280 nm (—) was determined. Glu-analysis (-●-) was performed on 250 µl aliquots of the effluent. Part of the effluent was pooled as indicated (A).

Fig. 2. Sephadex G-75 chromatography of partially purified BGP from calf bone. The BGP-fraction from the DEAE-cellulose chromatography (Fig. 1) was applied in 35 ml of 50 mM Tris-HCl buffer pH 8.0 to a column (5 cm x 100 cm) of Sephadex G-75, equilibrated and eluted at 4 °C with the Tris buffer. The flow rate was 60 ml/h and the effluent was collected in 13 ml fractions. The effluent was analysed by measurement of the absorbance at 280 nm (—) and by agarose gel electrophoresis of 10 µl aliquots. Part of the effluent was collected as indicated (A).

Fig. 3. DEAE-cellulose chromatography of partially purified calf bone BGP. The material obtained by Sephadex G-75 chromatography (Fig. 2) was dissolved in 20 ml of 50 mM Tris-HCl buffer pH 8.0, and applied to a column (5 cm x 20 cm) of DEAE-cellulose. The column, which had been equilibrated with 50 mM Tris-HCl buffer pH 8.0, was eluted with a linear gradient of NaCl (0 to 1 M in 2 l) in 50 mM Tris-HCl buffer pH 8.0, containing 10 mM CaCl_2 . The chromatography was performed at 4 °C, the flow rate was 60 ml/h and fractions of 15 ml were collected. The absorbance of the effluent at 280 nm (—) was measured and part of the effluent was pooled as indicated (A).

Fig. 4. HPLC of purified BGP. 10 µg of BGP in 40 µl starting solvent was injected to a µ-Bondapak C_{18} column (0.39 cm x 30 cm), which was eluted at 1.6 ml/min with a linear gradient from 84% solvent A : 16% solvent B to 50% solvent A : 50% solvent B, developed in 30 min. Solvent A: 0.02 M triethyl ammonium phosphate pH 4.0. Solvent B: acetonitril. The upper curve shows absorbance at 214 nm and the lower curve at 280 nm.

Table I. Purification of BGP from calf bone*.

Purification step	Dry weight (mg)	Gla content ($\mu\text{mol/g}$)	Yield of Gla (%)
Extraction	24,800	4.3	100
DEAE-cellulose chromatography	620	87.1	51
Sephadex G-75 chromatography	n.d.	n.d.	n.d.
DEAE-cellulose chromatography	47	360	16

*The starting material was 3000 g (wet weight) of coarsely crushed calf tibias, which contained a total of 450 μmol of Gla according to analysis of material obtained by extensive extraction.

n.d., not determined.

Table II. Amino acid analysis of purified BGP from calf bone.

Amino acid	Analysis (molar ratios)	According to sequence (ref. 3)
γ -Carboxyglutamic acid*	2.9	3
Hydroxyproline	0.8	1
Aspartic acid	6.1	6
Threonine	0.1	0
Serine	0.2	0
Glutamic acid**	3.0	3
Proline	6.2	6
Glycine	3.3	3
Alanine	4.4	4
Half-cystine	1.0	2
Valine	2.0	2
Methionine	0.0	0
Isoleucine	1.0	1
Leucine	5.0	5
Tyrosine	3.9	4
Phenylalanine	2.0	2
Histidine	1.9	2
Lysine	1.1	1
Arginine	3.0	3

* Determined after alkaline hydrolysis.

** Value obtained after acid hydrolysis minus value for γ -carboxyglutamic acid.

Table III. Sequence analysis of BGP purified from calf bone.

Cycle number	Identified PTH amino acid	Yield (nmol)	
1	Tyr	69.3	
2	Leu	45.1	
3	Asp	50.2	
4	His	n.d.	
5	Trp	n.d.	
6	Leu	35.7	
7	Gly	28.3	
8	Ala	33.3	
9	Hyp	n.d.	
10	Ala	25.2	
11	Pro	14.8	
12	Tyr	22.2	
13	Pro	23.6	
14	Asp	19.9	
15	Pro	14.2	
16	Leu	12.2	
17	Gla	1.3	Glu 2.0, Leu 4.2
18	Pro	9.1	
19	Lys	1.8	
20	Arg	n.d.	
21	Gla	1.3	Glu 1.5
22	Val	5.8	
23	-		
24	Gla	0.8	Glu 1.3
25	Leu	1.2	

Amount of peptide analysed: 0.49 mg (83 nmol).

Repetitive yield 91 % (Leu₂ - Leu₁₆).

n.d., not determined.

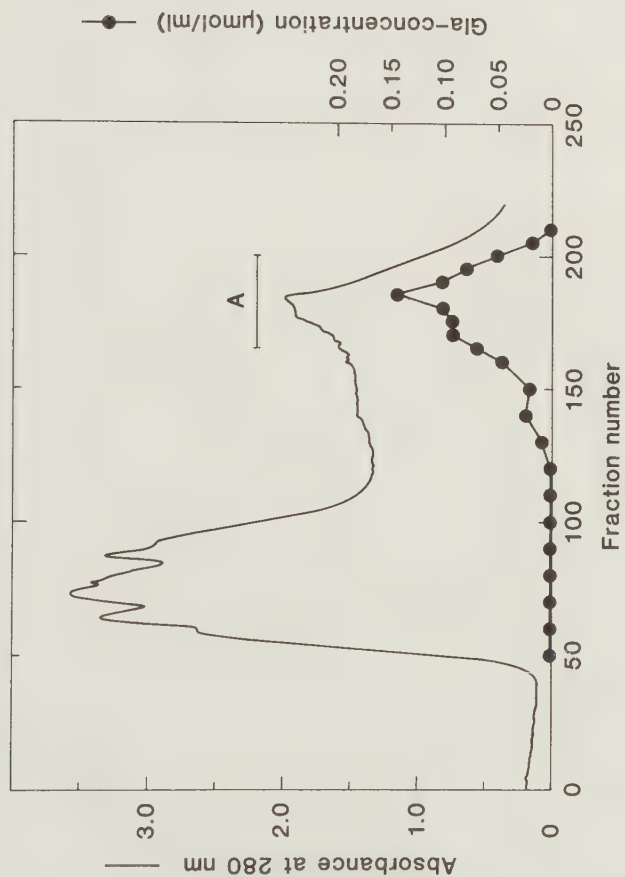


FIGURE S:1

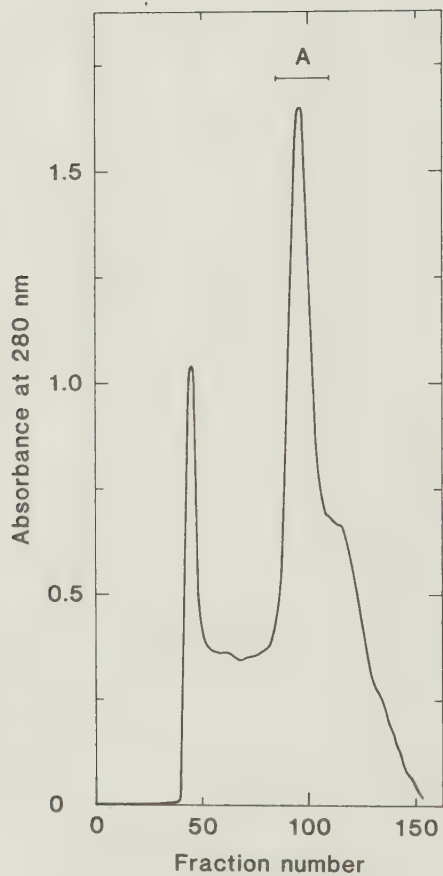


FIGURE S:2

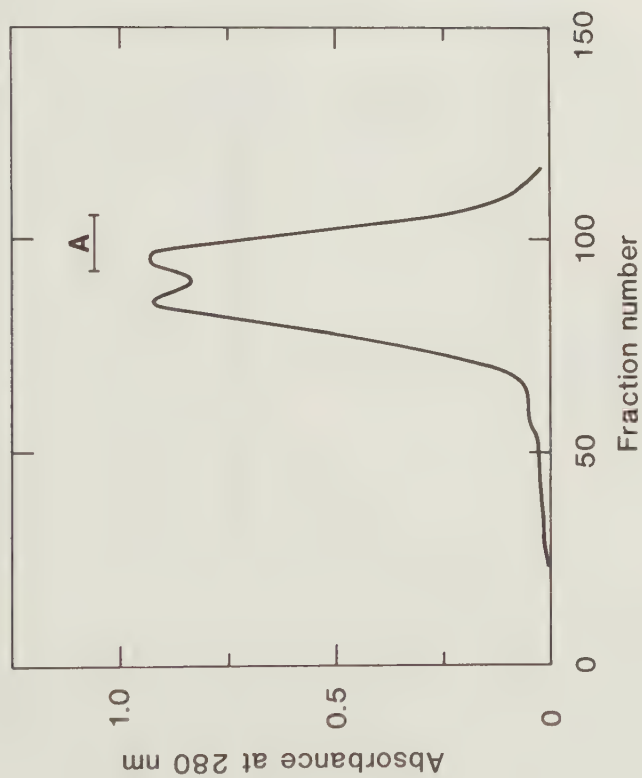


FIGURE S:3

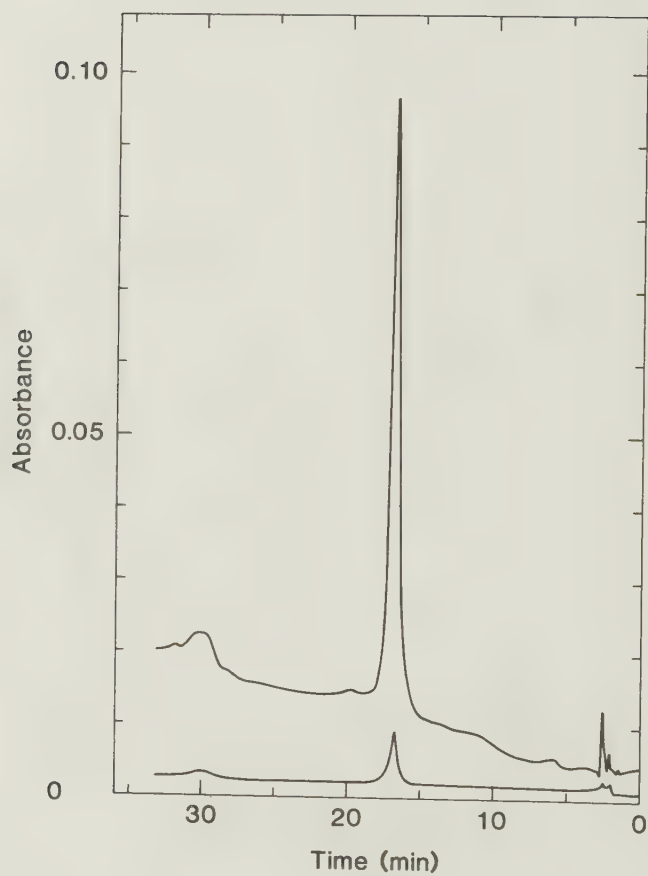


FIGURE S:4

Cation binding to bile salts. A ^{113}Cd NMR study.

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Summary

^{113}Cd NMR chemical shifts have been used to study the binding of Cd^{2+} ions to bile salts. $^{113}\text{Cd}^{2+}$ has, due to its magnetic properties and similarity in size with Ca^{2+} as well as preference of types of ligands, been successfully used in studies of calcium binding proteins. The present results can not be explained from a simple equilibrium model of 1:1 complexes between Cd^{2+} and bile salt. However, a model with both 1:1 and a 2:1 complexes between Cd^{2+} and taurocholate fits the experimental data well.

Introduction

Bile salts are important biological surfactants. They solubilise for instance monoglycerides and fatty acids in the intestine. In the gall-bladder, the bile salts form together with lecithin, mixed micelles in which cholesterol is solubilised. A review of the physico-chemical properties and the physiological function of bile salts has been made by Carey (1).

The interaction between bile salts and Ca^{2+} ions has been intensively discussed (2-7) since gall-stones have been found to consist not only of cholesterol but also of insoluble Ca^{2+} salts (8). The Ca^{2+} salts are mainly calcium carbonate, calcium phosphate, calcium bilirubinate or salts of fatty acids (9, 10). One reason for considering the interaction between bile salts and Ca^{2+} ions is that the presence of bile salt is thought to lower the Ca^{2+} activity and thereby prevent formation of gall-stones (3, 11).

The Ca^{2+} -bile salt interactions have been studied by means of Ca^{2+} -specific electrode (3-5), ultrafiltration (3) and equilibrium dialysis (2).

The crystal structure of a calcium salt of cholic acid has been determined (6). The structure was shown to contain bilayers of cholates with the hydrophobic part "sandwiched" between the polar groups interacting with Ca^{2+} ions and water molecules.

A Ca^{2+} -bile salt solution structure has also been suggested (4). In this complex the Ca^{2+} ion is proposed to be positioned between the ionic side chain group and an hydroxyl group on the cholanic acid ring.

From ^1H and ^{13}C NMR studies a 1:1 complex between Dy^{3+} and glycocholate was found (12, 13). In this solution structure, the Dy^{3+} ion binds to the carboxylate group of the bile salt.

^{113}Cd NMR chemical shifts have been demonstrated to be very sensitive to the type and number of ligands surrounding the Cd^{2+} ion (14, 15). The chemical shift range extends over 800 ppm (15).

The ionic radii of Cd^{2+} (0.97 Å) and Ca^{2+} (0.99 Å) are similar. ^{113}Cd NMR studies of proteins having Ca^{2+} substituted by Cd^{2+} has given a considerable amount of information (16, 17). ^{113}Cd chemical shifts has also been applied to polyelectrolyte systems, such as polystyrene sulfonate (18).

In this work we present an ^{113}Cd NMR study of Cd^{2+} -binding to some bile salts. The sodium salts of taurocholate (TC), taurodeoxycholate (TDC) and taurochenodeoxycholate (TCDC) are studied.

Materials and Methods

The bile salts were prepared, with slight modification, according to Norman (19) at the Department of Physiological Chemistry 4, Chemical Center, Lund, Sweden. The bile salts had been recrystallized at least twice.

Cadmium was added from a 0.1 M stock solution of $^{113}\text{Cd}(\text{ClO}_4)_2$ prepared from ^{113}CdO (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) with an isotopic enrichment of 96%.

^{113}Cd NMR spectra were recorded at 56.55 MHz on a laboratory built 6T spectrometer (20). ^{113}Cd NMR chemical shifts are given relative to the shift of an aqueous 0.1 M $\text{Cd}(\text{ClO}_4)_2$ solution with low-field shifts given positive values. All measurements were performed in water at a pH

around 7.2. The pH was adjusted using NaOH or HClO_4 .

Results and Discussion

The ^{113}Cd chemical shifts are known to be very sensitive to the type and number of ligands (14, 15). ^{113}Cd bound to sulfonate or carboxylate ligands give down-field and up-field chemical shifts, respectively. This is also the case for the taurine (SO_3^-) and glycine (COO^-) conjugated bile salts. However, the sodium glycocholate (NaGC) is difficult to study since cadmium glycocholate readily precipitates already at low Cd^{2+} concentrations.

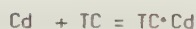
^{113}Cd NMR spectra were recorded for samples containing various concentrations of $^{113}\text{Cd}^{2+}$ and NaTC. It was then observed that for constant NaTC concentrations (1, 10 or 100 mM) there is for low cadmium concentration an increasing ^{113}Cd chemical shift with increasing cadmium concentration. At a cadmium concentration of 1–3 mM the ^{113}Cd shift passes a maximum, and for higher metal concentration the shift monotonously shifts towards the shift for free $^{113}\text{Cd}^{2+}$ -ions (Figure 1).

It is obvious that this concentration dependence of the ^{113}Cd NMR chemical shift does not fit with the formation of only a 1:1 complex, however, if we had not been using the low $^{113}\text{Cd}^{2+}$ concentrations ($< 3 \text{ mM}$) we could easily have drawn that conclusion. From the 1 mM TC solution an apparent binding constant of ca. 200 M^{-1} is obtained when only Cd^{2+} concentrations above 3 mM are considered. This fits quite well with a reported binding constant of 150 M^{-1} (4) for Ca^{2+} binding to monomeric TC (Figure 2A). The ^{113}Cd shift for the TC+Cd complex is ca. 15 ppm. A similar treatment of the data for the highest TC concentration (100 mM) does not work as well.

A more complex model of the cadmium – taurocholate association behavior

is needed. The addition of Cd^{2+} ions most likely changes the association behaviour of the bile salt. A lowering of the CMC value with the addition of Cd^{2+} ions is likely to occur (21). However, in the presence of Cd^{2+} , a TC concentration of 1 mM is assumed to be below CMC (CMC for NaTC has been reported to be 10 mM (22)).

Using the following model for Cd^{2+} binding to monomeric TC good agreement between observed and calculated ^{113}Cd shifts is obtained over the whole Cd^{2+} concentration range.



$$K_1 = \frac{[\text{TC} \cdot \text{Cd}]}{[\text{TC}][\text{Cd}]}$$



$$K_2 = \frac{[\text{TC} \cdot \text{Cd}_2]}{[\text{TC} \cdot \text{Cd}][\text{Cd}]}$$

The ^{113}Cd shift are calculated from

$$\delta^{\text{obs}} = p_{b1} \cdot \delta_1 + p_{b2} \cdot \delta_2$$

where p_{b1} and p_{b2} are the population of Cd^{2+} ions in the two complexes and δ_1 and δ_2 are the shifts of the ^{113}Cd nucleus in the two complexes. p_{b1} and p_{b2} can be obtained when all existing species are known. The free concentrations of Cd^{2+} and TC

$$[\text{Cd}] = 1 / (1 + K_1 \cdot [\text{TC}] + 2K_1 K_2 [\text{TC}] [\text{Cd}])$$

$$[\text{TC}] = 1 / (1 + K_1 [\text{Cd}] + K_1 K_2 [\text{Cd}]^2)$$

are best calculated through an iterative procedure for self-consistence. Figure 2B shows the results of such a calculation for a solution that was 1 mM in TC. The parameters used were $K_1=1500 \text{ M}^{-1}$, $K_2=300 \text{ M}^{-1}$, $\delta_1=2.6 \text{ ppm}$ and $\delta_2=14 \text{ ppm}$. We do not claim that this is the correct model for Cd^{2+} -ion binding to taurocholate, however, it shows that the data we have obtained can be explained in this way. Of course it is possible to use more complex models, this is, however, the most simple model that is able to explain our data. It also has some resemblance to what has been discussed by Moore et al for Ca^{2+} -ion binding to taurocholate (4).

When we tried to apply the same model calculation to the results obtained at higher TC concentrations (10 and 100 mM) we found that the data could not be fitted with this simple model and one has to go into more complex calculations. This is, however, outside the scope of this work. We can thus only state that the Cd^{2+} -ions will bind to both the monomeric taurocholate molecules and to the micelles. Both these modes of binding are sufficiently strong to affect the ^{113}Cd chemical shifts already at a Cd^{2+} -ion concentration below 1 mM.

In Figur 3 is shown the ^{113}Cd chemical shift as a function of $^{113}\text{Cd}^{2+}$ -ion concentration in the presence of 100 mM of taurocholate, taurodeoxycholate or taurochenodeoxycholate. We can here see that for all three "cholates" there are similar trends in the ^{113}Cd shift, with the most pronounced maximum for the NaTDC solution. It thus seems to be a general feature in the interaction between Cd^{2+} -ions and this class of molecules, and a complex model for this interaction has to be worked out.

Conclusion

There is a reasonably strong interaction between Cd^{2+} -ions and monomeric taurocholate. Even well below CMC for sodium taurocholate can the interaction not be described as the formation at only the 1:1 complex. At least two complexes with Cd/TC ratios of 1:1 and 2:1 have to be assumed. The stepwise binding constants for these two complexes are 1500 M^{-1} and 300 M^{-1} .

For the micellar solution is the interaction more complex with Cd^{2+} binding to monomers as well as to micelles effecting the ^{113}Cd NMR chemical shifts. More work is needed in order to get a better understanding of these interactions.

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Figure legends

- Figure 1 Observed ^{113}Cd NMR chemical shifts as a function of Cd^{2+} for 1.0 mM NaTC (+), 10 mM NaTC (o) and 100 mM NaTC (●).
- Figure 2 Calculated chemical shifts as a function of Cd^{2+} fitted to the experimental points of 1 mM NaTC assuming A) a simple equilibrium model of 1:1 complex between Cd^{2+} and TC^- and B) a model based on two different complexes namely with 1:1 and 2:1 of Cd^{2+} and TC^- ratios, respectively.
- Figure 3 Observed ^{113}Cd NMR chemical shifts as a function of Cd^{2+} for 100 mM NaTDC (+), 100 mM NaTC (o) and 100 mM NaTCDC (●).

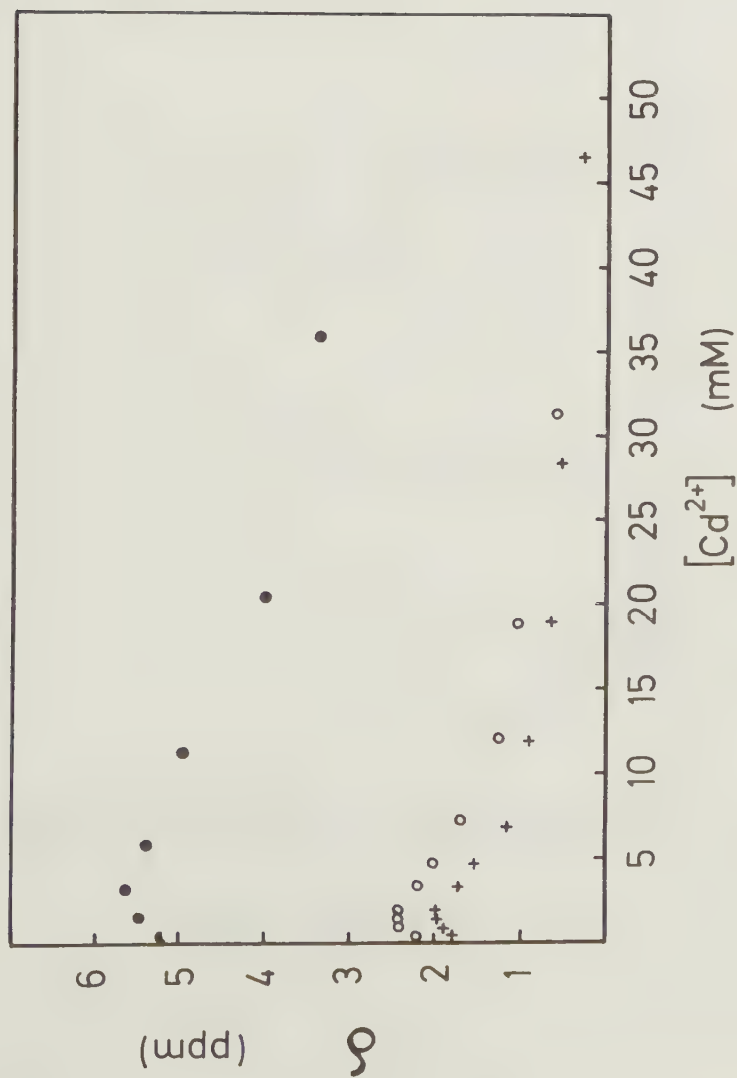


FIGURE 1

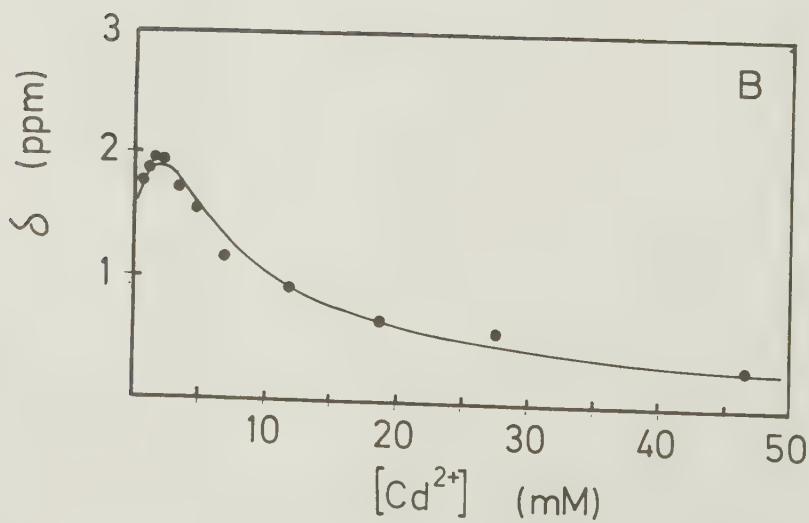
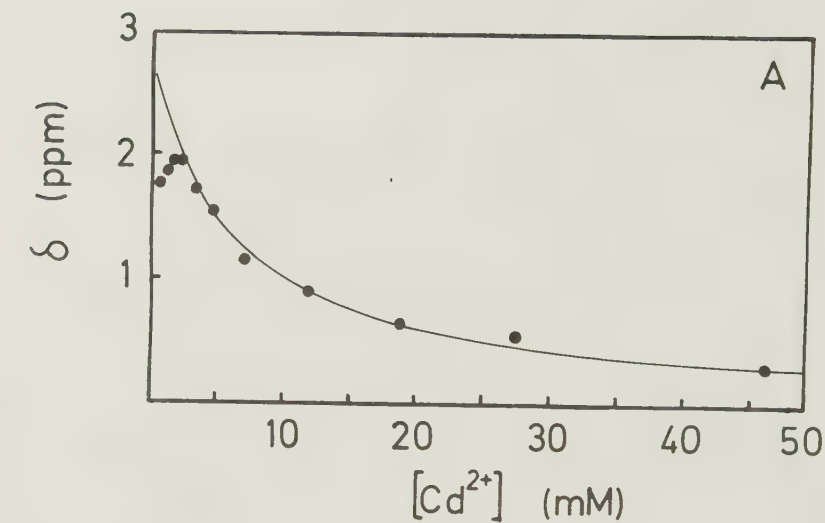


FIGURE 2

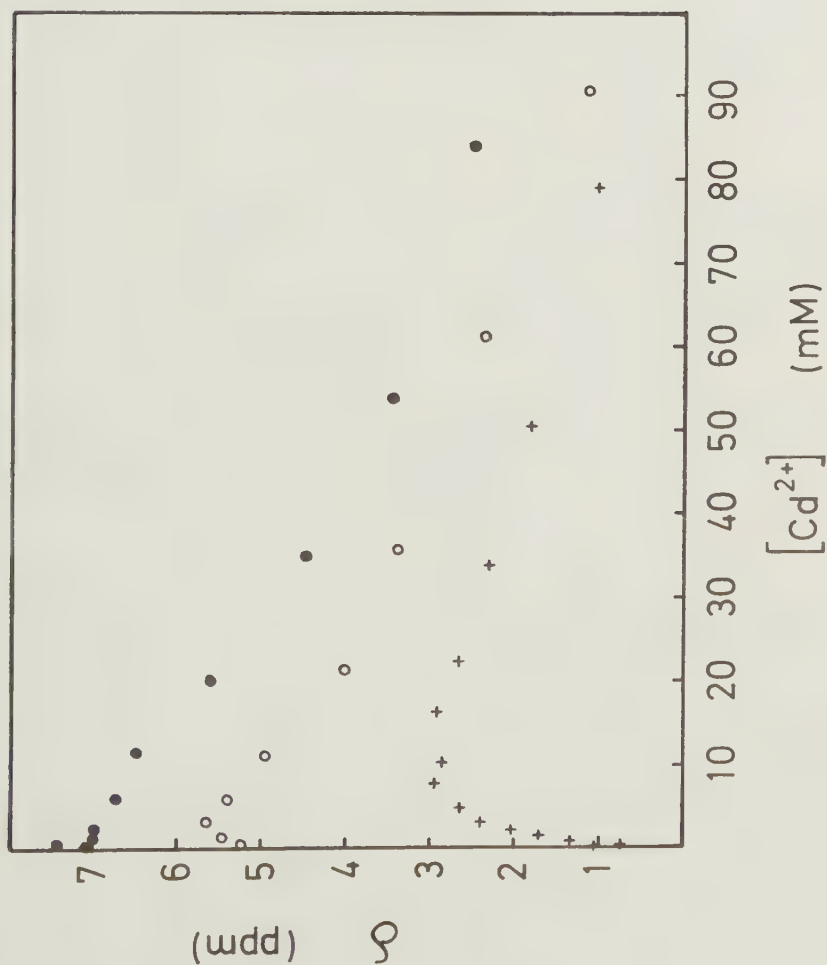


FIGURE 3

Micelles, Vesicles and Liquid Crystals in the Monoolein - Sodium Taurocholate - Water System. Phase behavior, NMR self-diffusion and Quasi-Elastic Light Scattering studies⁺

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Running title: The Monoolein-Sodium taurocholate-Water system studied by NMR and QLS.

Footnotes:

1. D_2O in this study was chosen for NMR experimental reasons. However, any water isotope effects would be insignificant (Carlfors & Stilbs, 1985). SDS micelles have been found to be somewhat bigger in D_2O than in H_2O but this is at high ionic strengths. (Chang, N.J. & Kaler, E.W. (1985))
2. Small (1971) summarized in a review article CMC-values that have been obtained for different bile salts. The large spread of the CMC-values not only depend on different techniques but also on the fact that it is difficult to define a distinct CMC value for especially trihydroxy bile salts as their association behavior differs from typical surfactant behavior. The CMC-values for bile salts have also been discussed by Carey and Small (1972), Mysels (1984) and Mukerjee et al. (1984). Kratochvil et al. (1983) have compared the aggregation behavior of NaTC and NaTDC.
3. In order to calculate P_b according to the Poisson-Boltzmann equation, the water layer thickness has also to be calculated. For a NaTC/MO molar ratio of 0.2 and 35% water, the calculated water layer is about 14 Å. This is in agreement with the value obtained from X-ray studies of monocaprylin-water (65-35 w%) which was found to be about 13 Å (Ekwall et al., 1968).
4. The transition temperature for chain melting has been determined from differential scanning calorimetry (DSC). For a sample containing 80 w% D_2O , 5w% NaTC and 15w% MO the crystallization of the chains occur at 8°C.

Abbreviations:

NMR, nuclear magnetic resonance;

QLS, quasielastic light-scattering;

MO, monoolein;

NaTC, sodium taurocholate;

NaTDC, sodium taurodeoxycholate;

NaC, sodium cholate

NaDC, sodium deoxycholate;

MO/NaTC, monoolein to sodium taurocholate molar ratio

l.c., liquid crystal;

NaAOT, sodium diethylhexylsulfosuccinate

DMPC, dimyristoyl phosphatidylcholine

Abstract

The mixed aggregation behavior in water of sodium taurocholate (NaTC) and monoolein (MO) was studied in isotropic solution and liquid crystalline phases. Different physico-chemical approaches such as phase diagram determination, ^2H and ^{23}Na quadrupole splittings, Fourier transform PGSE NMR self-diffusion and quasi elastic light scattering were used.

^2H NMR has been employed in combination with polarized light microscopy studies to determine the phase diagram of the sodium taurocholate-monoolein - water system. The diagram shows the existence of a large micellar phase, a lamellar and a cubic liquid crystalline phase. The lamellar l.c. phase has been examined from water deuterium and ^{23}Na quadrupole NMR splittings.

The changes in deuterium quadrupole splittings within the lamellar l.c. phase have been found to fit well to a model of constant hydration of the aggregate surface. The sodium quadrupole splittings are discussed in terms of fraction of bound ions which has been calculated from the Poisson-Boltzmann equation. The micellar region in the phase diagram has been further investigated by means of ^1H NMR self-diffusion and quasi elastic light scattering. Selfdiffusion coefficients for both NaTC and MO have been determined and show that at low MO/NaTC molar ratio (0.4) there is most probably coexistence of monomers, simple micelles and mixed micelles. At high MO/NaTC molar ratio (0.8) there is more likely a coexistence between mixed micelles and monomers. The results also suggest that the micellar aggregates are small in the micellar region. The combination of NMR self-diffusion, quasielastic light scattering and electron microscopy show that by diluting a micellar sample a two-phase region is reached. This two-phase region is between the micellar and the lamellar liquid crystalline phase. Two ways of interpreting self-diffusion coefficients in the pure sodium taurocholate system are presented.

The amphiphilic behavior of bile salts is well established and their physiochemical properties have been reviewed by Small (1971) and by Carey and Small (1972). Bile salts act as important solubilizers for mainly lecithin and cholesterol in the bile and for lipolytic products like monoglycerides and fatty acids in the small intestine. Much research has been focused on the importance and possible need of bile salt for the transport and absorption of lipids in the digesting gut. (Lindström et al., 1981). However, the processes which occur during transport and absorption of lipids are not fully understood and it is of great value to know more about the physical state of the content of the small intestine. The roles of bile acids in digestion and absorption have been discussed by Borgström et al. (1985). Lipid digestion and absorption has also been reviewed by Carey et al. (1983).

It is obvious that knowledge about phase diagrams of these systems become essential to the efforts to solve the questions above and other nearlying problems.

Several binary phase diagrams of a monoglyceride and water have been determined (Lutton, 1965; Larsson, 1967). Bile salts do not form liquid crystalline phases in water but sodium deoxycholate (Rich & Blow, 1958; Blow & Rich, 1960) do form gel type structures under certain circumstances. Also ternary phase diagrams with a bile salt as one of the components have been studied. Regarding the bile salt - monoglyceride - water systems there are to our knowledge only the phase diagram studies by Hofmann (1964), Lawrence (1964), Lawrence et al. (1967) and Larsson et al. (1978).

Information on molecular interactions in liquid crystalline systems can conveniently be obtained from deuterium and alkali ion NMR (Lindblom et al., 1976; Lindman et al., 1980). When quadrupolar nuclei (^2H , $I=1$; ^{23}Na ,

$I=3/2$) experience an anisotropic surrounding as in for instance lamellar and hexagonal liquid crystalline phases, the NMR spectrum shows splittings of the signals due to non-averaged quadrupolar interactions. Thus, by combining water deuteron and 23 -sodium NMR in the studies of liquid crystalline phase interactions between ions, water molecules and the aggregate surfaces are displayed.

In this paper the phase diagram of sodium taurocholate (NaTC)-monoolein (MO)-water (D_2O) is outlined. Structure formulae for NaTC and MO are shown in Figure 1. The phase diagram has been determined by means of deuterium NMR and polarizing microscopy. The lamellar liquid crystalline phase has been studied from deuteron and sodium- 23 quadrupole splittings as functions of water content and MO/NaTC molar ratios. The results are compared with a simple model of water binding. The fraction of bound ions have been calculated according to the Poisson-Boltzmann equation and the results are discussed together with the ^{23}Na quadrupole splittings.

The micellar region of the present system has been investigated in greater detail by NMR self-diffusion measurements and quasielastic light scattering.

The self-diffusion Fourier transform NMR, developed by Stilbs & Moseley (1980) enables simultaneous determination of self-diffusion coefficients for several components in the system. Thus, both bile salt and monoglyceride self-diffusion coefficients can be determined. The usefulness of this technique, as applied to amphiphilic systems, has been demonstrated by Stilbs (1983) and Lindman et al. (1981).

The applicability of quasielastic light scattering (QLS) to bile salt-lipid systems has been demonstrated by Mazer et al. (1979, 1980, 1984; Schurtenberger et al., 1983). QLS has in this work been used to study the effect of dilution on the aggregates in mixed micellar solutions of the monoolein-sodium taurocholate system.

Experimental Procedure

Materials. Sodium taurocholate was prepared, with slight modification, according to the procedure by Norman (1955) at the Department of Physiological Chemistry 4, Chemical Center, Lund, Sweden. The bile salt had been recrystallized at least twice.

1-Monoolein was purchased from Larodan Fine Chemicals, Malmö, Sweden. Purity was checked on thin-layer chromatography and gave only one spot when 2 mg was applied. D_2O of >99.7% isotopic purity was obtained from Norsk Hydro, Norway. The solvent used throughout this study was D_2O .

Sample preparation. Samples for phase diagram studies were prepared by weighing the three components directly in glass ampoules which were flame sealed and equilibrated. The ampoules were inserted in a 12-mm NMR tube for NMR measurements.

Sample preparation for NMR diffusion and QLS studies was made according to the co-precipitation method (Small et al., 1969). Appropriate amounts of bile salt and monoglyceride were dissolved in ethanol. The solvent was evaporated and D_2O ¹ was added to the dry mixture which then was carefully checked to be a clear solution prior to further dilution. The stock solutions contained 86 mg/ml of NaTC and MO content was varied from 11.4 mg/ml to 57 mg/ml to give MO/NaTC molar ratios of 0.2, 0.4, 0.6, 0.8 and 1.0. The dilutions were made by adding D_2O to the stock solutions to give the desired concentration. For QLS experiments the samples were equilibrated at $25 \pm 0.1^\circ C$ prior to investigation. At the time of measurement the samples were filtered for 3 minutes in a closed-circuit system in the light scattering cells with a peristaltic pump and Sartorius membrane filters of 0.2 μm pore size. The experimental temperature was kept at $25 \pm 0.1^\circ C$.

The NMR diffusion measurements were performed on samples that had previously been studied by QLS.

Methods

Quadrupole splittings: Deuterium and 23 -sodium NMR in the studies of lyotropic liquid crystals. The deuteron NMR spectra were recorded at 15.35 MHz using a modified Varian XL-100-15 spectrometer operating in the Fourier transform mode. An external proton lock was used. Sodium NMR was run at 67.43 MHz on a home-built Fourier transform spectrometer equipped with an Oxford Instruments 6-T wide bore magnet. All spectra were recorded at 28°C.

The quadrupole splitting method provides information about the liquid crystalline structure and on interactions between counterions and amphiphiles and between counterions and water. These aspects of amphiphilic systems have been reviewed by Lindman (1983) so here will only be given a short recapitulation of the use of quadrupole nuclei for the study of liquid crystalline phases.

Nuclei with spin $I \geq 1$ possess an electric quadrupole moment which interacts with electric field gradients at the nuclear site. In isotropic systems, like a micellar solution, the quadrupole interaction is averaged to zero due to fast motion. In an anisotropic surrounding the quadrupolar interaction causes a splitting of the signal into $2I$ equidistant peaks. The frequency separation of two nearby resonances is called the quadrupole splitting and denoted Δ .

Examples of ^2H and ^{23}Na NMR spectra are shown in Figure 2. A sample with microcrystallites randomly oriented with respect to the magnetic field gives a "powder-type" spectrum and the quadrupole splitting is given by

$$\Delta = |\nu_Q \cdot S| \quad (1)$$

ν_Q , for ^2H , is $3/4\chi$ where χ is the quadrupole coupling constant i.e. the product of the quadrupole moment of the nucleus and the field gradient. S is the order parameter which describes the time-averaged orientation of a molecular vector with respect to the symmetry axis of the phase.

Due to fast exchange in the system, the observed splitting, Δ , is an average over the different sites, i ,

$$\Delta = \left| \sum_i p_i \nu_Q^i \cdot S_i \right| \quad (2)$$

where p_i is the fraction of ions or molecules in site i . On the other hand, water exchange between different microcrystallites is typically slow and spectra from different phases will become superimposed on each other. Other factors being the same, the quadrupole splitting of a lamellar phase is twice that of a hexagonal phase, due to rapid water diffusion around the rods (Wennerström et al., 1974). In practise, a comparison between different phases presupposes a correction for differences in water content.

Self-diffusion measurements with NMR. Proton self-diffusion measurements were made at the Department of Physical Chemistry, Uppsala, on a Jeol FX-100 Fourier transform NMR spectrometer, using internal D_2O lock. The sample temperature was kept at 25 ± 0.2 °C. Initially, ^1H T_1 relaxation times were determined with the standard $180^\circ - \tau - 90^\circ$ inversion recovery procedure to ascertain a pulse repetition of at least $5T_1$.

The technique used in this study is ^1H Fourier transform Pulsed Gradient Spin Echo NMR (FT ^1H -PGSE NMR) as developed by Stilbs and Moseley (1980). This technique enables the diffusion coefficients for all components in the system to be determined simultaneously. This is possible if the T_2 relaxation time is not too short and the concentrations are kept within a reasonable range. When measuring on bile salt, the short T_2 -values do become a problem.

In the experiment, a spin echo is produced by a $90^\circ\text{-}\tau\text{-}180^\circ\text{-}\tau$ sequence. A field gradient (G) is applied during a time (δ) before and after the 180° pulse.

Due to diffusion the spins will not rephase completely resulting in an attenuated spin echo. The second half of the spin echo is Fourier transformed to give the contributions from the different signals in the spectrum. The dependence of the peak heights, A_i , on the length of the field gradient pulses, δ , follows the relation:

$$A_i \propto \exp(-(\gamma G \delta)^2 D_i (\Delta - \delta/3)) \quad (3)$$

where G is the field gradient strength, δ is the length of the gradient pulse, γ is the magnetogyric ratio for the nucleus studied, D_i is the diffusion coefficient and Δ is the time interval between the gradient pulses. G is determined from an experiment where D is known, Δ was kept constant and D was then obtained from the relation between A and δ .

Figure 3a shows the ^1H NMR spectra after a 90° pulse and a $90^\circ\text{-}\tau\text{-}180^\circ\text{-}\tau$ pulse sequence. The H₂O peak in the latter has disappeared due to fast water diffusion. The signals that correspond to the sterol nuclei have disappeared due to very short T_2 -values. This makes it possible to measure MD diffusion by following the peak from the methylene protons. The other peaks left after the $90^\circ\text{-}\tau\text{-}180^\circ\text{-}\tau$ pulse sequence correspond to the 18 methyl protons and protons of the 25 and 26 carbons of NaTC.

Figure 3b shows the spectral appearance during the diffusion experiment and the decrease in signal intensities as the length of the gradient pulse, δ , increases.

All diffusion data have been calculated using a program, UNIFIT, written by Peter Stilbs, Institute of Physical Chemistry, Uppsala.

Quasielastic light scattering. The light scattering apparatus consist of an argon ion laser (Spectra Physics, model 171, $\lambda = 5145 \text{ \AA}$), a digital autocorrelator (Malvern, K 8023, 96 channels), on-line data analysis on a Nova 3 computer and a temperature controlled scattering cell holder. Details are given elsewhere (Haller, 1980). Experiments were performed at 90° scattering angle and at a temperature of $25 \pm 0.1^\circ \text{C}$. Additional experiments were made on a commercial Malvern spectrometer. The mean collective diffusion coefficient, D_c , was determined by means of a cumulant analysis of the intensity auto correlation function (Koppel, 1972). From D_c an apparent mean hydrodynamic radius, $\bar{R}_H$, was calculated using the Stokes-Einstein relation. The data analysis procedure has been described previously (Schurtenberger et al., 1983).

Results and Discussion

The phase diagram. The ternary phase diagram of Sodium taurocholate - Monoolein - Water determined from deuterium NMR and polarized light microscopy studies is shown in Figure 4. The principles of the ^2H NMR method for phase diagram determination have been described in previous publications (Khan et al., 1982; Lindman, 1983).

Only the one-phase regions are outlined and the diagram shows the existence of a large isotropic solution phase, a lamellar l.c. phase and a cubic l.c. phase.

The solubility of sodium taurocholate in water is about 58%. The aqueous solutions can solubilize large amounts of monoolein in isotropic solution (the maximal MO content in the isotropic solution phase is around 60%). The solubility of monoolein in water is very low and the binary system of monoolein and water shows at room temperature the existence of two l.c. phases, one cubic and one lamellar (Lutton, 1966; Krog et al., 1984). While the cubic

phase can only take up 4w% of NaTC the lamellar phase has the capability to incorporate up to about 22w% of NaTC. The latter phase has capability for a rather high content of water and swells to about 55 w% water. The deuterium NMR spectra and polarized microscopy suggest in addition the existence of a hexagonal l.c. phase but a one-phase region of this phase has not been established. Figure 2c shows a water deuteron NMR spectrum which demonstrates the coexistence of an isotropic solution phase, a hexagonal l.c. phase and a lamellar l.c. phase. The one phase region of the hexagonal liquid crystalline phase is apparently very small and exists probably at a water content of about 50w% in the region between the isotropic solution phase and the lamellar l.c. phase. The deuterium quadrupole splittings that correspond to the hexagonal phase are less than 100 Hz and consequently difficult to observe when there is a large isotropic peak in the spectrum and a large amount of lamellar l.c. phase in the sample.

It is also worth mentioning that at even higher water content (around 60 w%) in the "multi-phase" region, between the lamellar l.c. phase and the water-corner, the D_2O NMR spectra show line shapes similar to those found for a "ribbon" phase. (Chidichimo et al., 1982; 1984). In this concentration region a few samples also show very intense blue colour when visualized in polarized light. Furthermore, the texture of these samples when studied in polarized light microscopy do not unambiguously show the presence of the lamellar or hexagonal l.c. phases.

Samples with very low water content become difficult to study due to long equilibration times. At, 20% water, for instance, the samples in the isotropic solution phase are very viscous. The long equilibration times for lamellar l.c. samples at low water content are evident from changes in line shape of water deuteron quadrupole splittings with time.

The structure of the cubic monoolein-water phase has been studied by X-ray diffraction and NMR. (Lindblom et al., 1979). The structure is proposed

to be built up by lamellar bilayer units which are connected in a three-dimensional network. According to Hyde et al. (1984) there are two modifications of the structure depending on the water content. In the phase diagram presented in this work the notation cubic l.c. is used without any further implications of phase boundaries for different structures.

Considering that the monoolein is the structure forming lipid in these systems it is interesting to compare the effects when either NaTC, cholesterol or a protein, like lysozyme, is added to the binary MO-water cubic phase.

From the phase diagram in Figure 4 is seen that only about 4w% NaTC can be incorporated in the cubic phase. On the other hand, approximately 15w% of cholesterol can be solubilized (Larsson et al., 1978). In a study of the incorporation of proteins it has been found that about 35w% of lysozyme can exist in the cubic phase (Ericsson et al., 1983).

The solubility of cholesterol in water is very low. Bile salts are amphiphilic molecules but with surfactant characteristics that differ from "normal" longchained surfactants (for instance, bile salts are not known to form any liquid crystalline structures in water). Lysozyme is a small, basic protein ($M_r = 14600$, $pI = 11.0$). The hydrophobic cholesterol molecule is likely to be located in between the hydrocarbon chains of the monoolein. The effect of cholesterol on lipid bilayers has been studied from NMR by Rothman & Engelman (1972) and Stockton & Smith (1976). In these studies the alkyl chain order parameter was found to increase with the cholesterol concentration. The lysozyme molecule is situated in the water channels of the cubic phase and does therefore not influence very much the packing of the monoolein. Consequently a large amount of protein can be accommodated by the cubic monoolein - water phase. The sodium taurocholate on the other hand, affects the cubic structure clearly since only 4w% can be added before it is disrupted. This effect can be explained if the bile salt molecule is assumed to

be lying flat in the bilayer of monoolein resulting in both repulsive interactions in the aggregate surface and changes in the aggregate structure.

This model of packing was suggested by Ulmius et al. (1982) to exist in the sodium cholate - lecithin-water system on the basis of a decrease in the alkyl chain order parameter as cholate concentration was increased.

Besides a comparison between systems where the effect of a third component (NaTC, cholesterol and lysozyme) on the cubic MO-water phase it is also of interest to compare bile salt systems where the structure forming amphiphile is changed. This can be made under the assumption that the di- and trihydroxy bile salt do not differ greatly in terms of possible ways of packing in lipid matrices. Two such amphiphiles which have been studied are diethylhexylsulfo-succinate (Aerosol OT, AOT) and lecithin. AOT and lecithin are ionic and zwitterionic, respectively, and they have both two alkyl chains.

The phase diagram of NaDC-NaAOT-water has been determined by La Mesa et al. (1984) and show similarities with the system of NaTC-MO-water. The AOT molecule has a quite large hydrophobic volume with its branched alkyl chains compared to MO and is therefore more disposed to form extended lamellae. A quite large lamellar phase is consequently found in the NaDC-AOT-water system. No other liquid crystalline phase is found between the lamellar and the isotropic solution phases.

The phase diagram of sodium cholate (NaC) - lecithin - water determined by Small et al. (1966) also shows a large lamellar l.c. phase. Between the lamellar and the L_1 phase, there are in this system a hexagonal and a cubic l.c. phase.

As regards an interpretation of the observed phase diagram in terms of molecular packing and interactions we are not in a position for a more deep-going analysis. However, we note that the introduction of taurocholate ions into the monoolein bilayers will influence both inter- and intralamellar interactions. The acquired charges of the bilayer lead to interbilayer repul-

sions and thus increased swelling and incorporation of water. As result of both electrostatic and steric effects, the latter more so in the model of Ulmuis et al. (1982) than in the model of Small (1971), the a $1/v$ term in the theory of Israelachvili et al. (1976) increases with increasing NaTC content (a is the headgroup area, l is the chain length and v is the volume of the hydrocarbon chain or chains). Thus, one expects a transition from planar aggregates to aggregates with a higher curvature as is observed.

Hydration from water deuteron quadrupole splittings. Water quadrupole splittings have been shown to be a useful experimental parameter to study hydration in liquid crystalline phases (Wennerström et al., 1976; Persson & Lindman, 1975). Consequently, to study hydration the lamellar liquid crystalline phase in the MD-NaTCwater system was investigated by means of ^2H quadrupole splittings.

It is normally instructive to divide water molecules into two states, i.e. "bound" and "free". The "bound water" includes water that is hydrogen bonded to the aggregate surface and water surrounding the bound sodium ions. "Free" water molecules are to be considered as bulk molecules. The exchange rate between the two sites is fast so the quadrupole splitting can be described as an average of the different sites i.e. free and bound. The quadrupole splitting, Δ , is then described as

$$\Delta = |p_f \Delta_f + p_b \Delta_b| \quad (4)$$

where p_f and p_b are fractions of molecules in free and bound states, respectively.

Expression (4) can be rewritten as

$$\Delta_{D_2O} = |\Delta_f + (n \cdot X_A / X_{D_2O}) (\Delta_b - \Delta_f)| \quad (5)$$

where n is an average hydration number and X_A and X_{D_2O} are mole fractions of amphiphile and water respectively. If it is assumed that water added above a certain level only changes the amount of free water, then a plot of

Δ_{D_2O} against the molar ratio of amphiphile (here NaTC and MO) to water would give a straight line with a slope of $n(\Delta_b - \Delta_f)$ (and an intercept, Δ_f).

Such a plot is shown in Figure 5a. (The samples studied lie along line 1 in Figure 4). As can be seen in the figure, the experimental points lie along a straight line with zero intercept. This gives support for a model of "bound" and "free" water molecules with the latter having a negligible Δ -value. Furthermore, n and Δ_b appear to be constant over a wide range of water concentrations, strongly indicating that the area per polar headgroup remains invariant.

The magnitude of the splitting at the "bound" site Δ_b is

$$\Delta_b = \frac{3}{4} |\chi S_b| \quad (6)$$

where χ is the quadrupole coupling constant and S_b the order parameter (See Methods).

In the interpretation of water deuterium quadrupole splittings in lyotropic liquid crystals the assumption of χ being insensitive to the sample composition can be made (Persson and Lindman, 1975). χ for 2H in D_2O has been experimentally determined by Halle & Wennerström (1981) to be 222 kHz. According to Figure 5 and Figure 4, n is estimated to be in the range 4-10. A calculation of S_b according to equation (6) gives $S_b \approx 0.005$.

Counterion binding from 23 -sodium quadrupole splittings. Sodium 23 NMR quadrupole splittings can give information about counterion binding (Lindblom et al., 1976; Lindblom et al., 1978). Since the ions in the bulk solution experience isotropic surroundings, Δ_f can be neglected and expression (4) is reduced to

$$\Delta = |\rho_b \Delta_b| \quad (7)$$

Thus, the observed quadrupole splitting contains information about the

fraction of ions at the aggregates surface provided Δ_b is constant.

However, Δ_b for the MO-NaTC system can be expressed as

$$\Delta_b = X_{MO} \Delta_{MO} + (1 - X_{MO}) \Delta_{NaTC} \quad (8)$$

where X_{MO} is the mole fraction MO in the lamella.

Figure 6a shows the observed ^{23}Na quadrupole splitting as a function of NaTC to MO molar ratio at constant water of 35% (see line 2 in Figure 4). The quadrupole splitting decreases as the NaTC/MO molar ratio increases. The fraction of bound ions, p_b , is expected to increase as the NaTC content increases (due to increased charge density) therefore the observed decrease is referred to as changes in Δ_b . The number of "bound ions" may be calculated using the Poisson-Boltzmann equation if it is assumed that the ions within a specified length, ΔL , from the aggregated surface may be described as bound to the surface. This length is usually assumed to be the same as the diameter of a water molecule i.e. $3 \text{ \AA}$, but this value is not a critical value as may be seen in the work by Engström & Wennerström (1978).

From the calculated p_b values the Δ_{MO} and Δ_{NaTC} were determined through a fitting procedure using equations (7) and (9). The result of the fit is shown as the full-drawn line in Figure 6a. This fit gave $\Delta_{MO} = 13.6 \text{ kHz}$ and $\Delta_{NaTC} = -23 \text{ kHz}$. Therefore, the decrease in ^{23}Na quadrupole splittings as the NaTC/MO molar ratio increases is mainly due to changes in Δ_b i.e. changes in counterion location at the aggregate surface. The calculated p_b changes from 0.49 to 0.60 as the NaTC/MO molar ratio goes from 0.1 to 0.3.³

Figure 6b shows ^{23}Na quadrupole splittings as a function of water content in the lamellar phase. The NaTC/MO molar ratio is kept constant at 0.2 (See line 1 in Figure 4). As can be seen in the figure, the quadrupole splitting decreases as water content increases.

In order to see whether this decrease in splitting is due to a decrease in the fraction bound ions, p_b was calculated from the Poisson-Boltzmann equation. In the calculation of P_b a correction is made assuming dissociation of bile salt from the lamella as water content increases (Jönsson, 1981). The concentration of NaTC in the water between the lamellae as a function of total water content is shown in Figure 6c. In this calculation the highest NaTC concentration is set to 10 mM. The fulldrawn line in Figure 6b shows the calculated decrease in the ^{23}Na quadrupole splitting as water content increases. The calculated curve reproduces the trend of the experimental values quite well but lies above the experimental points since the starting values are taken from the fit in Figure 6a and do not therefore fully agree with the experimental point at 35.5% D_2O in figure 6b. A possible explanation of the (relatively small) discrepancy between the two curves is that, besides a decrease in p_b as water content increases there is a change in the aggregate surface with water content. However, this is not supported by the results in Figure 5a showing the hydration of the surfaces staying constant.

NMR self-diffusion studies. The micellar phase of the NaTC-MO- D_2O system has been further investigated by means of self-diffusion studies using the ^1H FT-PGSE NMR technique (Stilbs, 1983). Both MO and NaTC diffusion coefficients were determined simultaneously. Due to the very low solubility of MO in water (10^{-6} M, Kåre Larsson, personal communication), the concentration of monomeric MO can be neglected, MO diffusion coefficient corresponds to the diffusion of mixed MO-TC micelles. Since fast exchange conditions can be assumed, the bile salt diffusion is a weighted average of the monomers and micelles (simple and mixed micelles) according to

$$C_{\text{tot}} \cdot D_{\text{obs}} = C_{\text{mon}} \cdot D_{\text{mon}} + C_{\text{mic}} \cdot D_{\text{mic}} \quad (2)$$

where C_{tot} is total bile salt concentration. C_{mon} the monomer concentration and C_{mic} the micellar bile salt concentration. D_{obs} is the observed diffusion coefficient, D_{mon} can be determined in a separate experiment thus allowing D_{mic} to be calculated.

The diffusion coefficient for monomeric taurocholate was determined at 4.0 mM NaTC to be $33.0 \cdot 10^{-11} \text{ m}^2 \text{ s}^{-1}$. In Table I a comparison is made between this value of monomer diffusion and values obtained from the open-ended capillary method. The fact that the values in Table I are within reasonable agreement suggests that the CMC-value for taurocholate lies above 5 mM.

Figure 7a shows the observed bile salt diffusion as a function of the total bile salt concentration at different MO/NaTC molar ratios. At constant MO/NaTC, the diffusion coefficient decreases as the total concentration increases. The diffusion coefficient also decreases as MO/NaTC molar ratio increases.

Included in Figure 7a is also the observed NaTC diffusion when no MO is present. The observed diffusion coefficient is in that case a weighted average of monomer and simple micellar diffusion according to (9). The results at 20 and 40 mM NaTC are discussed since at these concentrations intermicellar interaction effects on the diffusion coefficient can most probably be neglected. (Kratohvil et al., 1983). Reported values of R_H for the simple TC-micelles lie around 10 Å (Mazer et al., 1979; Lindheimer et al., 1981; Schurtenberger & Lindman, 1985). The hydrodynamic radius and the diffusion coefficient are related through the Stokes-Einstein relation, $R_H = kT/6\eta D$ (for spherical particles), where η is the viscosity of the medium. Assuming a hydrodynamic radius of 10 Å for the micelle gives a monomer concentration of about 13 mM according to equation (9). This value for C_{mon} is somewhat higher than the value of 10 mM reported by Roda et al. (1983)². On the other hand, assuming C_{mon} to be 10 mM gives a micellar diffusion

coefficient which corresponds to a hydrodynamic radius of about 8 Å. At 40 mM NaTC, the value of 10 mM for C_{mon} gives $R_H = 11.4$ Å. Thus, if we assume that the monomer concentration is constant at 10 mM then the hydrodynamic radius changes from about 8 to 11 Å as the total bile salt concentration goes from 20 to 40 mM. The association behavior for especially trihydroxy bile salts has been observed to be gradual (Ekwall, 1957; Norman, 1960; Lindman et al., 1984; Kratochvil, 1983).

If we on the other hand assume a constant micellar size with $R_H = 10$ Å this gives that the monomer concentration according to our results would change from about 13 to 4.5 when total concentration goes from 20 to 40 mM NaTC. It has been shown by Lindman et al. (1984) that the monomer concentration does not change very much as total concentration increases. In Figure 7a, two points are included to illustrate what the observed NaTC diffusion coefficient would be if we assume $C_{\text{mon}} = 10$ mM and $R_H = 10$ Å for the micelles.

Our results suggest then that there is a growth of the simple TC micelle but it is apparently small.

Figure 7b shows the observed MO and thus also the mixed micellar diffusion coefficient as a function of the total bile salt concentration for different MO/NaTC molar ratios. The diffusion coefficient of the mixed micelles decreases as the MO content increases. D_{MO} also decreases with increasing NaTC concentration. This decrease is probably due to both an increase in size of the mixed micelle and an increase in interactions. For MO/NaTC = 0.2 there is possibly an increase in D_{MO} as NaTC concentration increases. However, these points will not be discussed here due to inadequate accuracy. The excluded volume effect is expected to produce a decrease in the self-diffusion coefficient (Lekkerkerker & Dhont, 1984). This means a decrease by about 10% at 160 mM NaTC when MO/NaTC increases from 0 to 1. However, the observed

decrease in the D_{MO} is larger which is consequently attributed to an increase in micellar size. If the observed NaTC diffusion in the mixed system is interpreted to be due to monomeric and mixed micellar NaTC then the difference between MO and NaTC diffusion coefficients provides information on the monomer concentration. For the discussion, the results for two different MO/NaTC ratios are compared. For MO/NaTC = 0.8, the assumption of only monomers and mixed micelles leads to a monomer concentration of about 8 mM. This value is somewhat higher than the values that have been reported for the NaTC-MO mixed system (Hofmann, 1963; Lindheimer, 1983).

For MO/NaTC = 0.4 the same assumption i.e. that NaTC exists either as monomers or in mixed micelles, gives a monomer concentration of about 12 mM which seems too high. However, using a more reasonable lower monomer concentration the difference between NaTC and MO diffusion coefficients, can be explained assuming the coexistence of monomers, simple micelles and mixed micelles.

Therefore, our results are consistent with the appearance of monomers and mixed micelles at MO/NaTC = 0.8 and a coexistence between monomers, simple micelles and mixed micelles at MO/NaTC = 0.4. For the mixed system of sodium taurocholate and lecithin it has been suggested that simple and mixed micelles coexist when the lecithin to NaTC molar ratio is below 0.6 while above this ratio there are mixed micelles in equilibrium with monomers only (Mazer et al., 1980).

Quasielastic light scattering studies. (QLS). In Figure 8 the mean hydrodynamic radius, $\bar{R}_H$ obtained from the QLS diffusion coefficient, is plotted as a function of NaTC concentration at two different MO/NaTC molar ratios. The experiment was performed by diluting a micellar stock solution with D_2O . The highest concentration of NaTC in these experiments is 160 mM.

The apparent mean hydrodynamic radius ($\bar{R}_H$) goes from 10 Å to 30 Å within the concentration range of 25-160 mM NaTC (for both MO/NaTC = 0.2 and 0.8). A very dramatic change in $\bar{R}_H$ occurs at concentrations below 21.9 mM for MO/NaTC = 0.8 and below 15.4 mM NaTC for MO/NaTC = 0.2. Upon dilution beyond this transition point, $\bar{R}_H$ decreases from a maximum of approximately 700 Å to approximately 200 Å at low concentrations.

A similar behavior has been found in bile salt-lecithin systems (Schurtenberger et al., 1985) where a micelle-to-vesicle transition occurs at concentrations below the mixed micellar phase limit. The dependence of the vesicle size upon the concentration has been analysed in terms of a simple phenomenological model which led to a quantitative relationship between the vesicle size and the amount of bile salt in the bilayer.

MO-NaTC solutions, in which large particles were found, were investigated by means of freeze-fracture electron microscopy. These studies revealed that vesicles are formed which are unilamellar.

Samples were also measured after different equilibration times. Samples containing small micellar aggregates show no time dependence. However, solutions at low concentrations containing large aggregates do vary with time. In general, we found that close to the transition point, $\bar{R}_H$ increased only slightly. (For example at 21.4 mM NaTC and MO/NaTC = 0.8, $\bar{R}_H$ increases from 360 Å after 24 hours equilibration time to 380 Å after 144 hours). This relative stability is most likely due to the repulsive electrostatic interaction between the vesicles arising from the presence of TC in the vesicle bilayer. At low concentrations, well below the transition point, $\bar{R}_H$ increased substantially with time. (For example, at 25 mM NaTC and MO/NaTC = 0.8, $\bar{R}_H$ increases from 190 Å after 24 hours equilibration time to 480 Å after 144 hours). At these low concentrations the number of bile salt ions in the bilayer (and thus surface charge density) should be substantially lower

due to a partitioning of the bile salt between the vesicles and the aqueous phase. The growth of the vesicles under these conditions is similar to the behavior of DMPC at temperatures below the phase transition of the aliphatic chains (Sornette and Ostrowsky, 1983). However, for the MO-NaTC system, a temperature of 25°C is well above the phase transition temperature for the chains.⁴

The electron microscopy studies of solutions with 2.5 mM NaTC and MO/NaTC = 0.8 at increasing equilibration time showed that a small but increasing fraction of large vesicles is formed. However, the majority of lipid is still present as small unilamellar vesicles with $R_H \approx 110$ Å. The large dependence of $\bar{R}_H$ upon the equilibration time can be understood from the fact that R_H is a weighted average (Z-average) over all particles present in the solution (Schurtenberger and Hauser, 1984).

It is interesting to compare the results from the QLS and the NMR diffusion studies. Both techniques reveal the existence of small micelles at concentrations within the micellar phase region. The differences between diffusion coefficients obtained from QLS and NMR and their small changes with concentration are most likely due to a combination of intermicellar interaction effects and changes in micellar sizes. However, clear differences between QLS and NMR results can be observed at concentrations below the transition point. The NMR self-diffusion measurements show the existence of small mixed micelles at 20 mM NaTC and MO/NaTC = 0.8 whereas QLS provides a $\bar{R}_H \approx 700$ Å under these conditions. A careful analysis of the intensity autocorrelation function indicates a bimodal size distribution with coexisting small ($R_H = 30$ Å) and large ($R_H = 750$ Å) particles. (Schurtenberger et al., 1985).

In the bile salt - lecithin system a substantial micellar growth is found close to the micellar phase limit. However, in the MO-NaTC system we do not find any indication for a similar micellar growth. Instead we find an abrupt

separation into two different types of aggregates (mixed micelles and vesicles). This means that a two-phase region is reached which lie in between the micellar and the lamellar liquid crystalline phases.

Our findings are in clear disagreement with previously published results on micellar growth in monoolein-bile salt systems (Lindheimer et al., 1983). In these studies, the results from tracer diffusion measurements were interpreted in terms of the mixed disc model proposed for bile salt - lecithin systems (Mazer et al., 1980). This discrepancy is probably due to the fact that at low bile salt concentrations one has to be aware of the phase limit of the mixed micellar system.

Concluding remarks

In this work polarized light microscopy, deuterium and sodium NMR have been used to evaluate the phase diagram of monoolein(MO)-sodium taurocholate(NaTC)-heavy water. We have found three one-phase regions: an isotropic homogenous micellar phase, a lamellar liquid crystal and a cubic liquid crystal phase. NMR and polarizing microscopy also suggest the existence of a hexagonal l.c. phase but it is apparently very small.

The lamellar liquid crystalline phase has been more thoroughly studied with respect to the NaTC/MO molar ratio and the water content, respectively. Water deuteron NMR quadrupole splittings suggest that the hydration of the aggregate surface stays invariant within the lamellar l.c. phase. Changes in Sodium-23 NMR quadrupole splittings in the lamellar l.c. phase have been interpreted to be due to both changes of the fraction bound sodium ions and changes in the aggregate surface.

The micellar phase of the system has been further investigated by means of diffusion studies using pulsed gradient spin echo ^1H NMR and quasi-elastic light scattering (QLS). By combining these two methods complementary information has been obtained. The NMR experiments suggest that there is a

small growth of the simple TC micelle with increasing NaTC concentration. In the mixed monoolein-sodium taurocholate system there are monomers, simple and mixed micelles coexisting at low MO/NaTC molar ratio (0.4) while at high MO/NaTC ratio (0.8) there are only monomers and mixed micelles coexisting.

From QLS and electron microscopy a micelle-to-vesicle transition was observed.

Bile salts and monoglycerides in water represent an interesting system in many respects. It offers a comparison of the phase behavior with common amphiphilic systems. It is also of physiologic relevance since it can be used as a model system in studies of fat digestion and absorption in the intestine.

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Table I Comparison between monomeric bile salt self-diffusion coefficients obtained from the tracer-technique and NMR. The data which were obtained from the open-ended capillary method were made in phosphate buffer at pH 7.4, 0.15 M NaCl at 37°C. These values have been converted to 25°C and heavy water.

References	$D_{\text{mon}}, 10^{-11} \text{ m}^2 \text{ s}^{-1}$	
Woodford (1969)	35.4	(5.0 mM NaTC)
Lindheimer et al. (1981)	37.3	(5.0 mM NaTC)
Sherrill et al. (1973)	32.1	(0.25 mM NaTC)
This work	33.0	(4.0 mM NaTC)

FIGURE LEGENDS

- Figure 1 Structure formulae of 1-monoolein and sodium taurocholate.
- Figure 2 Typical ^2H and ^{23}Na NMR spectra of samples from different parts of the phase diagram. Weight % of MO, NaTC, D_2O are given within parenthesis.
- a) lamellar l.c. + isotropic phase (48.9, 5.1, 46.0) b) lamellar l.c. phase (41.6, 9.9, 48.5) c) lamellar l.c. + hexagonal l.c. + isotropic phase (36.2, 12.8, 51.0) d) same sample as in b.
- Figure 3a ^1H NMR spectra for a sample of 160 mM NaTC and 32 mM MO. (MO/NaTC = 0.2). The lower spectrum shows spectral appearance after a 90° pulse. The upper spectrum shows spectral appearance after a $90^\circ - \tau - 180^\circ - \tau$ pulse sequence. The gradient pulse used here is too long to allow the HDO peak to be observed.
- Figure 3b The ^1H NMR spectrum at the top shows the peaks that correspond to NaTC after the $90^\circ - \tau - 180^\circ - \tau$ pulse sequence. Signal intensities as functions of the length of the applied pulse gradient (δ) are shown for a sample where NaTC = 40 mM and MO/NaTC = 0.6. The HDO peak is not observed here due to very fast diffusion.
- Figure 4 The ternary phase diagram of NaTC-MO- D_2O determined at 28° . The lines indicate the samples which have been studied from water deuteron and sodium -23 quadrupole splittings at constant MO/NaTC ratio of 0.2 (line 1) and at constant water content of 35% (line 2).
- Figure 5 Water deuteron quadrupole splittings as a function of molar ratio of amphiphile (NaTC and MO) to D_2O . See line 1 in the phase diagram in Figure 4.

Figure 6a Sodium-23 quadrupole splittings as a function of NaTC/MO molar ratio at constant water content of 35%. See line 2 in the phase diagram in Figure 4. The full drawn line is the result from a calculation of p_b according to the Poisson-Boltzmann equation and a fit of expression (8). From the fitting procedure $\Delta_{MO} = 13.6$ kHz and $\Delta_{NaTC} = -23$ kHz were obtained.

Figure 6b Sodium-23 quadrupole splitting as a function of water content at a constant NaTC/MO molar ratio of 0.2. The full drawn line represents a calculated quadrupole splitting with the assumption of constant Δ_b (values of Δ_{MO} and Δ_{NaTC} were obtained from the fit in a.). p_b was calculated from the Poisson-Boltzmann equation with the correction of increased amount of NaTC in the water layer as water content increases.

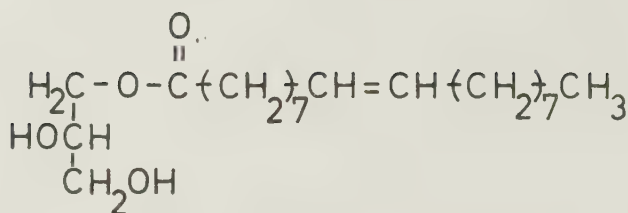
Figure 6c The calculated NaTC concentration in the water layer as a function of water content.

Figure 7a The observed NaTC self-diffusion coefficient as a function of NaTC concentration at different MO/NaTC molar ratios. MO/NaTC = 0 (●), 0.2 (◐) 0.4 (▲), 0.6 (+), 0.8 (■) and 1.0 (■). Included in the figure are two points for MO/NaTC = 0 at NaTC = 20 and 40 mM which correspond to a calculated NaTC self-diffusion coefficient assuming a micellar hydrodynamic radius of 10 Å and a monomer concentration of 10 mM. Full drawn lines are just drawn through the experimental points as an help to the eye.

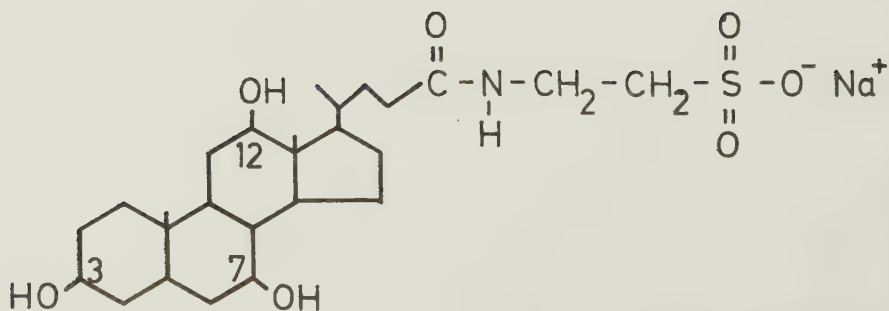
Figure 7b Measured MO self-diffusion, which represents mixed micellar diffusion. For MO/NaTC = 1.0 the sample at 20 mM NaTC was not a homogeneous solution therefore there are only three NaTC concentrations that have been studied at this molar ratio. Error bars for MO/NaTC = 0.4 are included.

Figure 8 The mean hydrodynamic radius, $\bar{R}_H$ obtained from QLS collective diffusion coefficients, as a function of bile salt concentration at MO/NaTC molar ratio of 0.2 and 0.8.

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1-MONOOLEIN



SODIUM TAUROCHOLATE

FIGURE 1

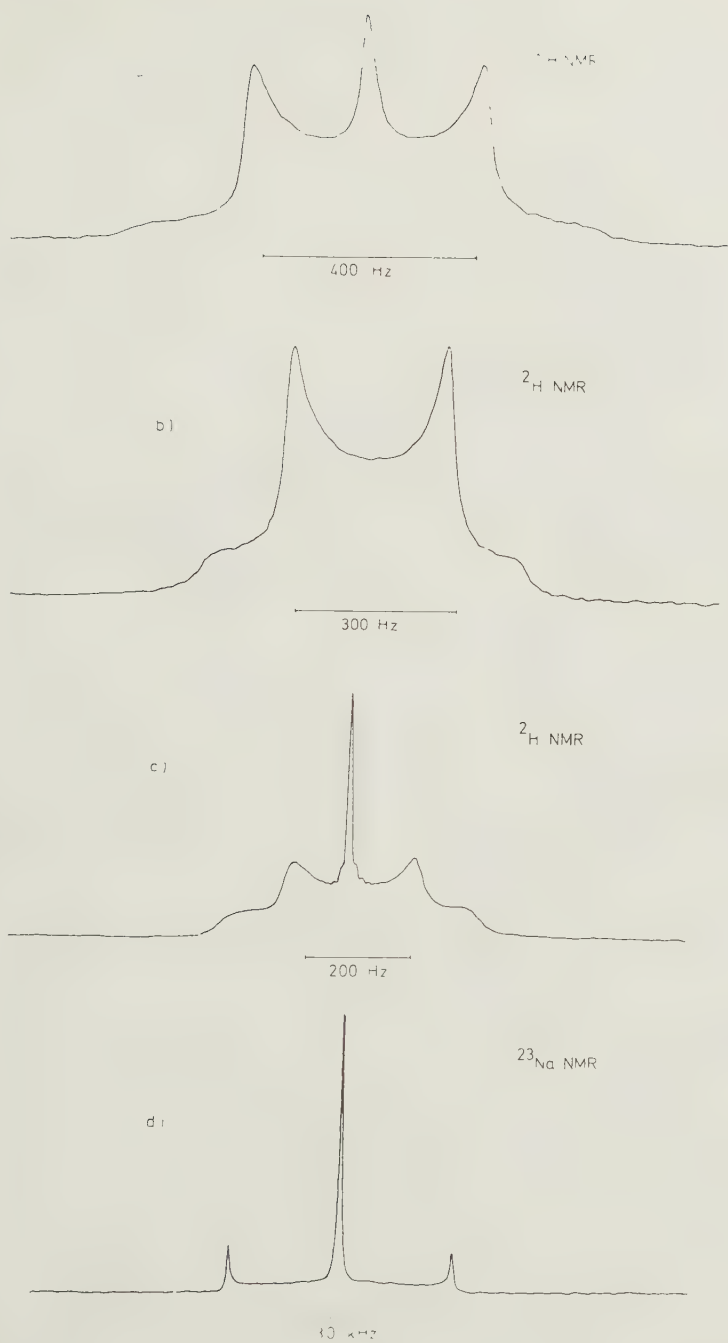


FIGURE 2

a

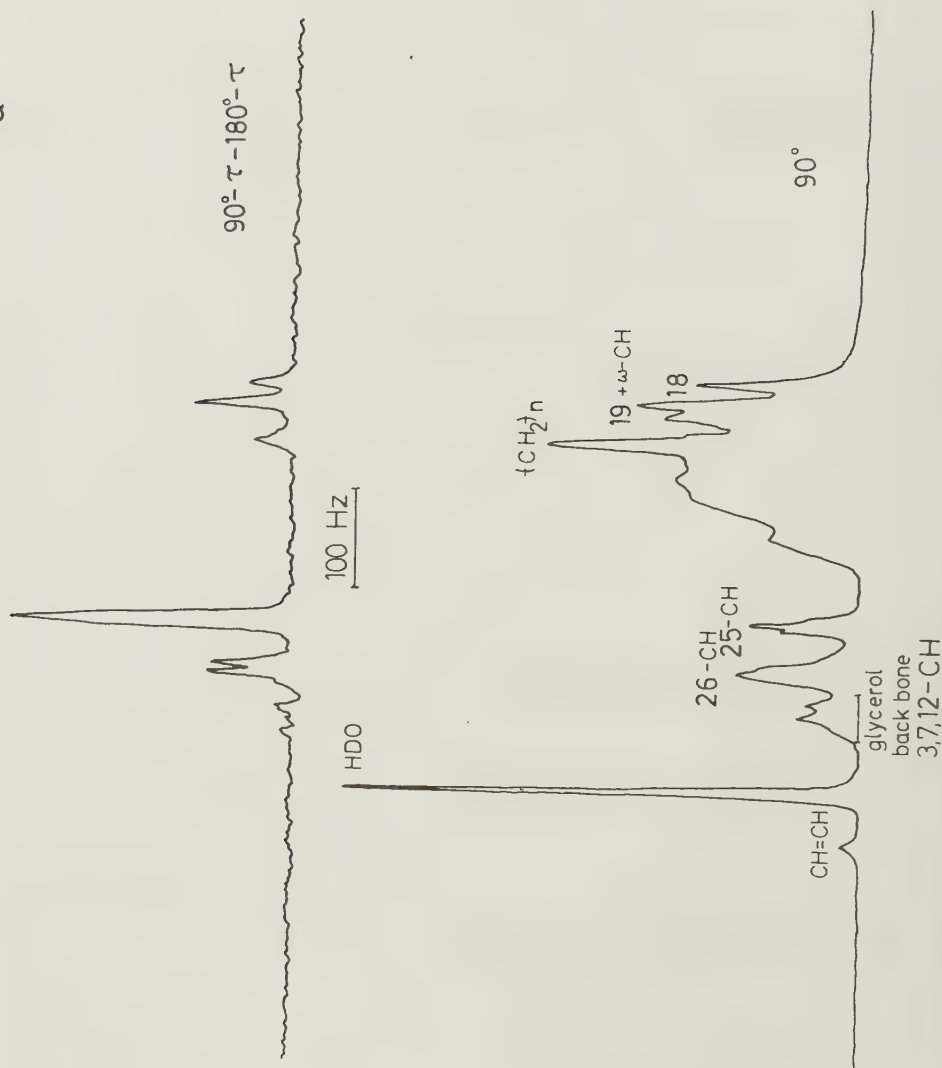


FIGURE 3a

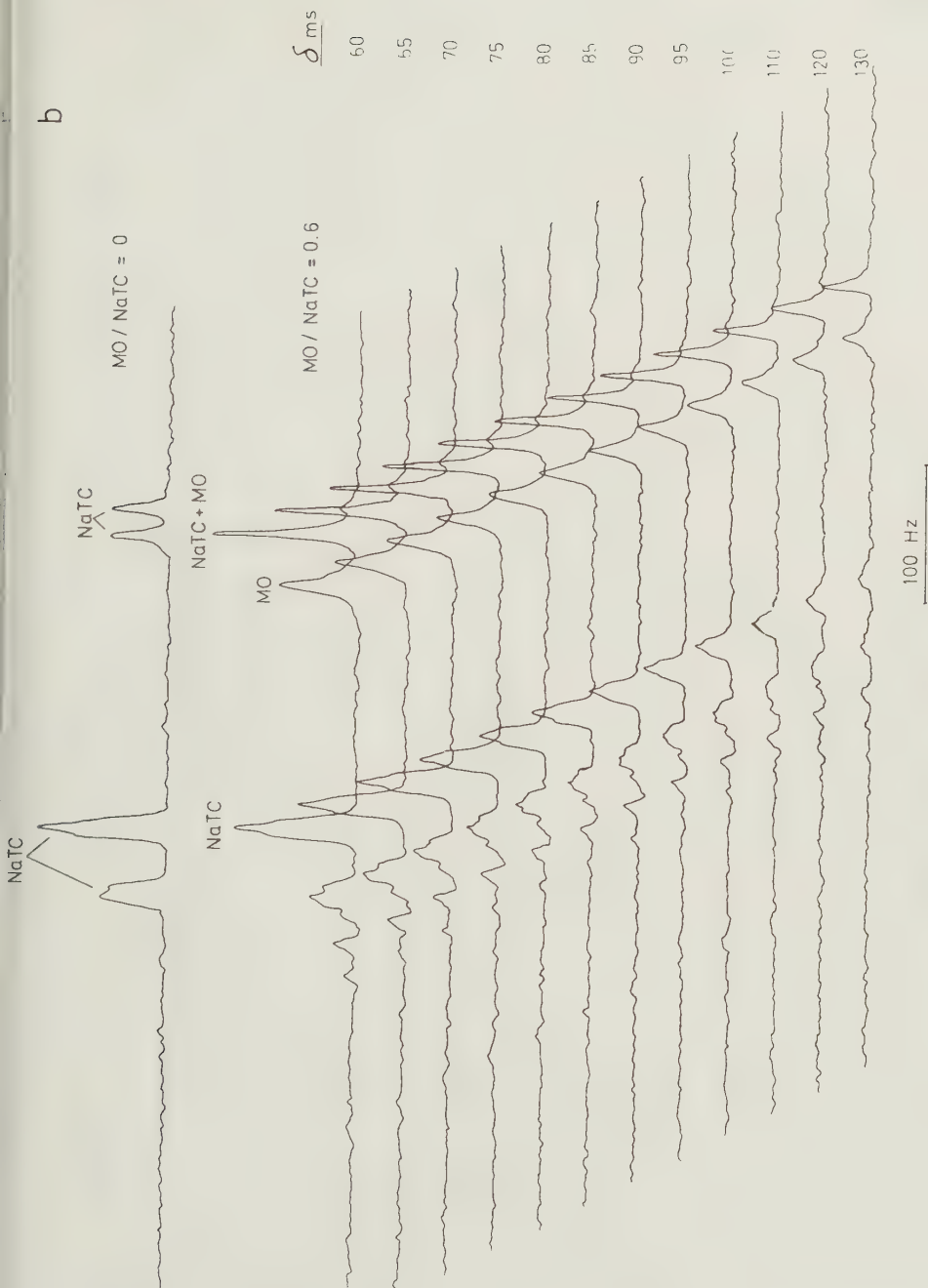


FIGURE 3b

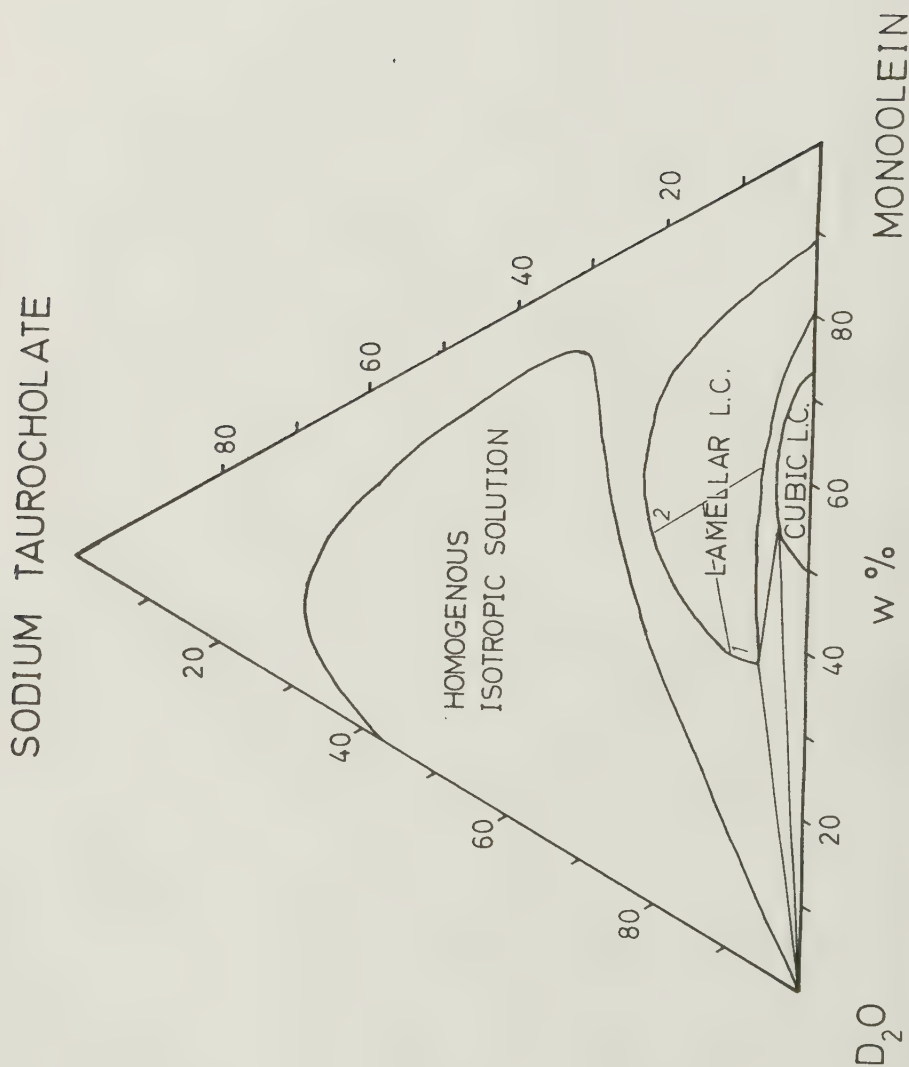


FIGURE 4

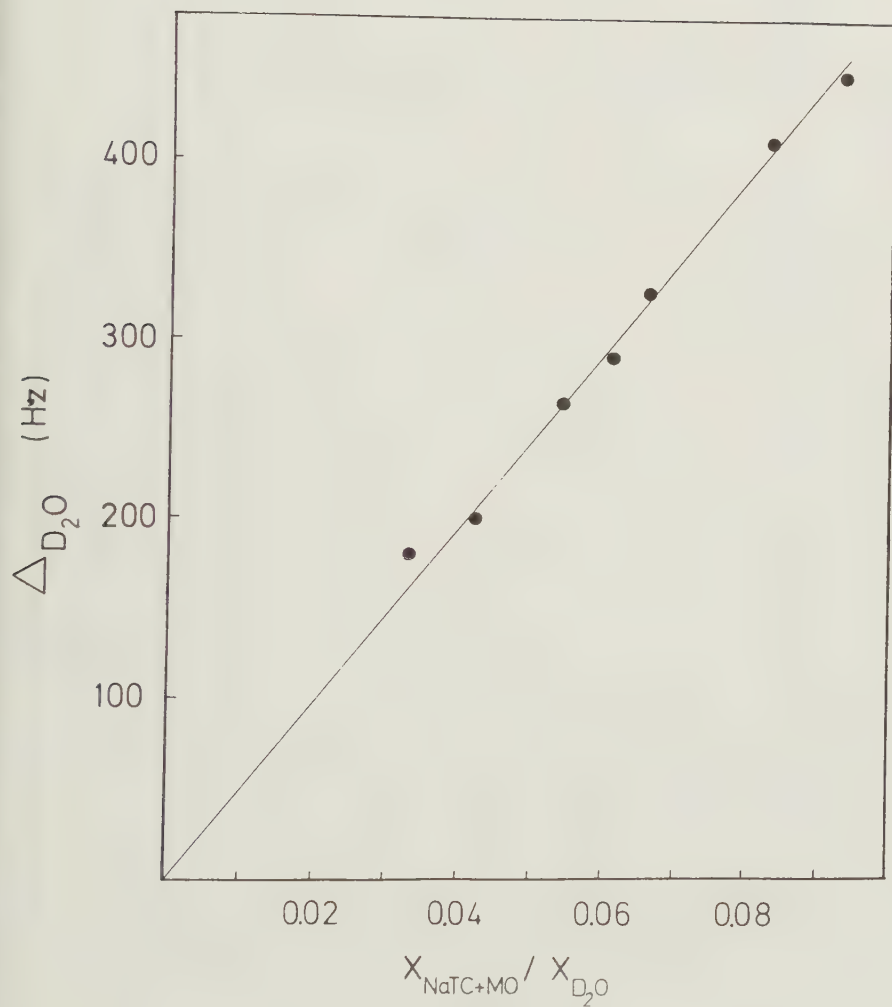


FIGURE 5

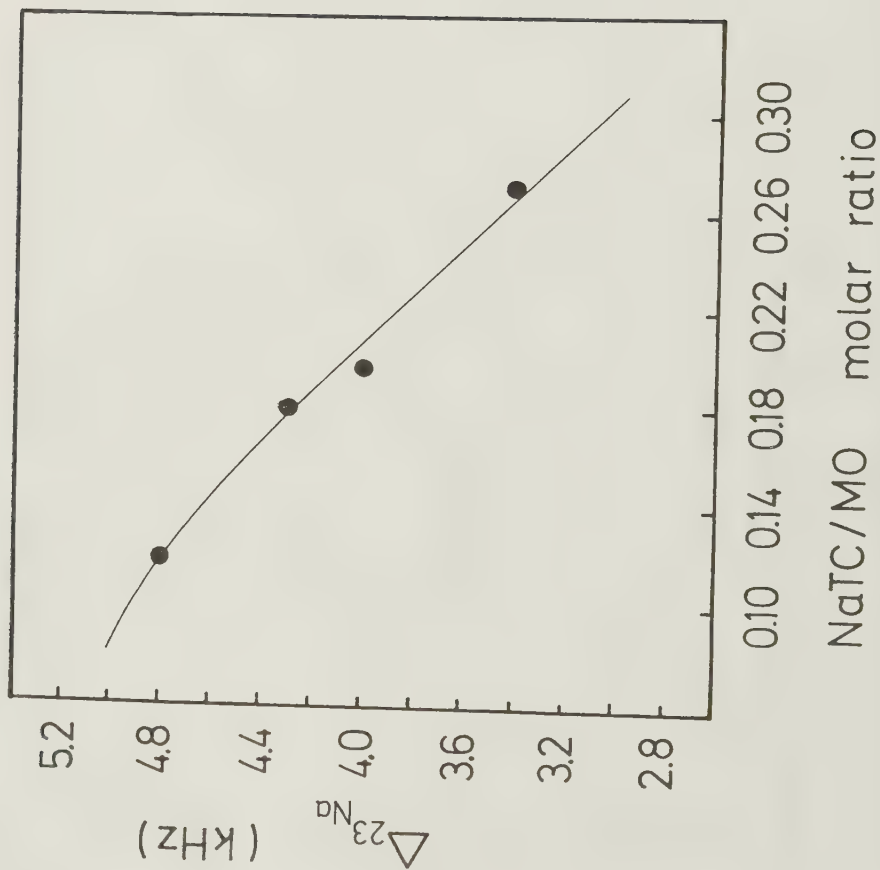


FIGURE 6a

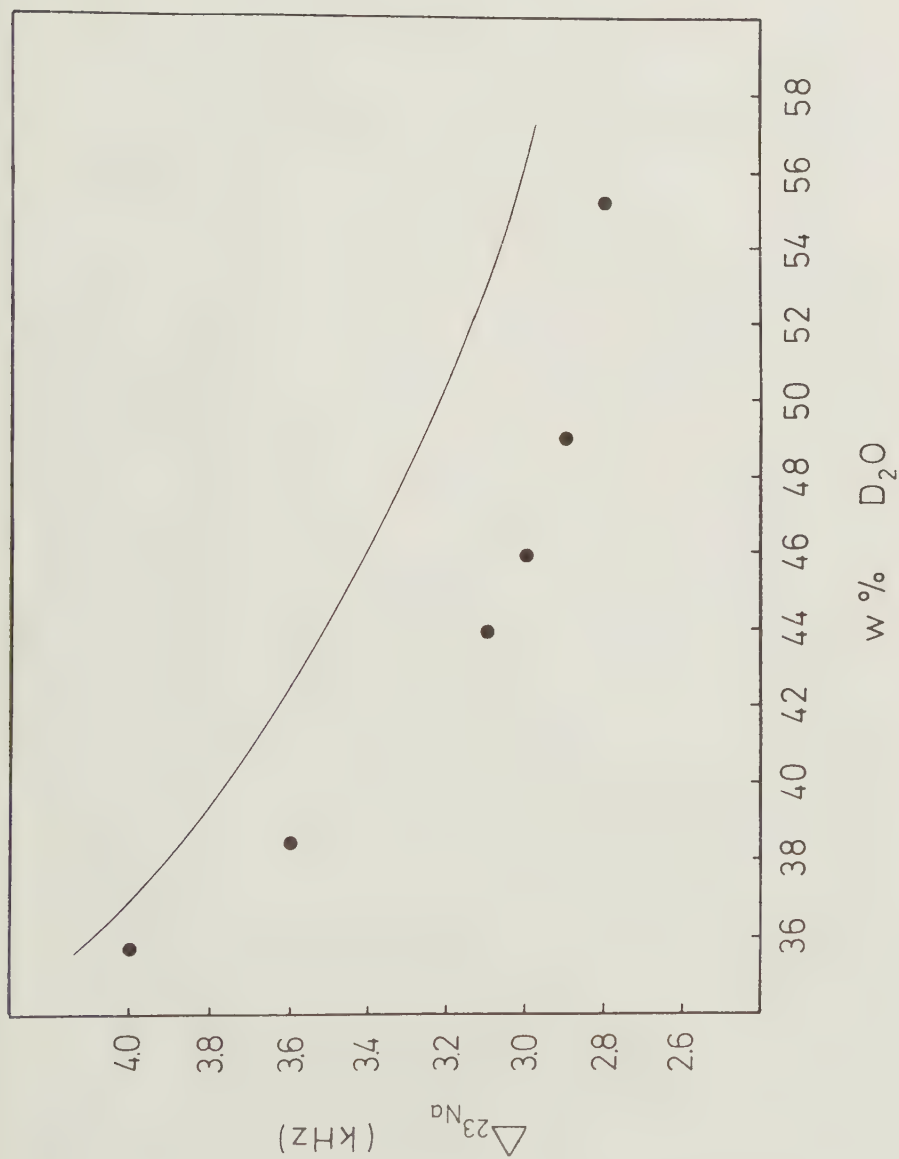


FIGURE 6b

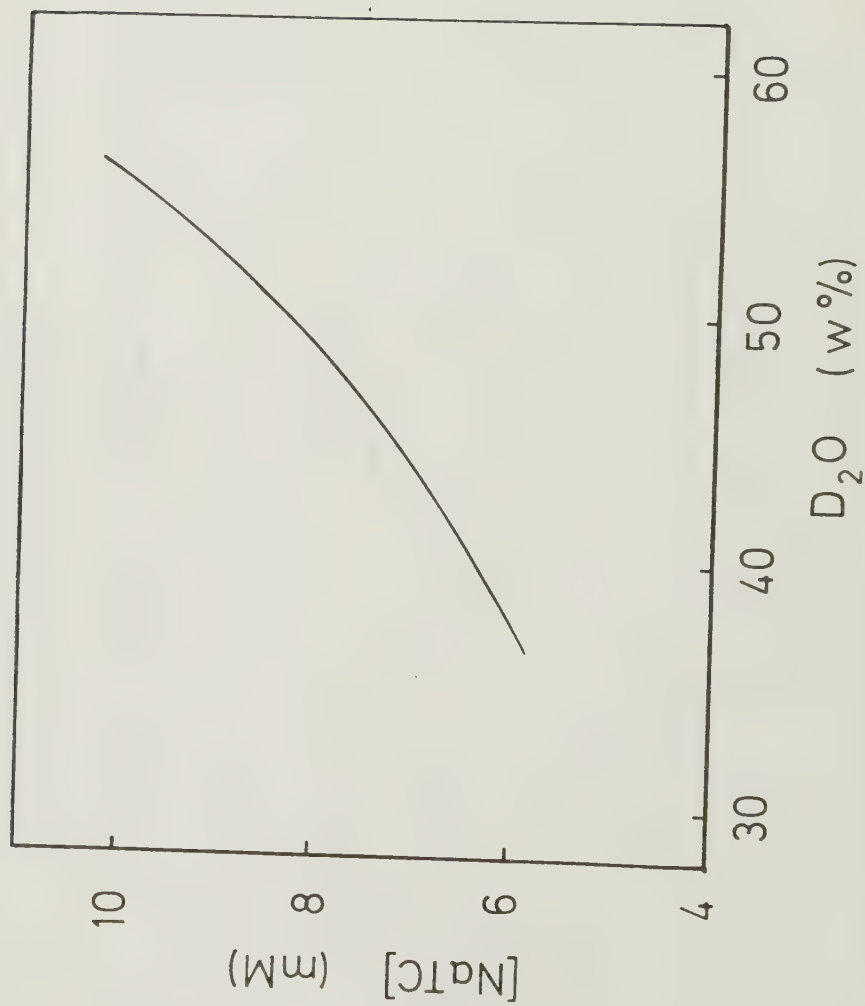


FIGURE 6c

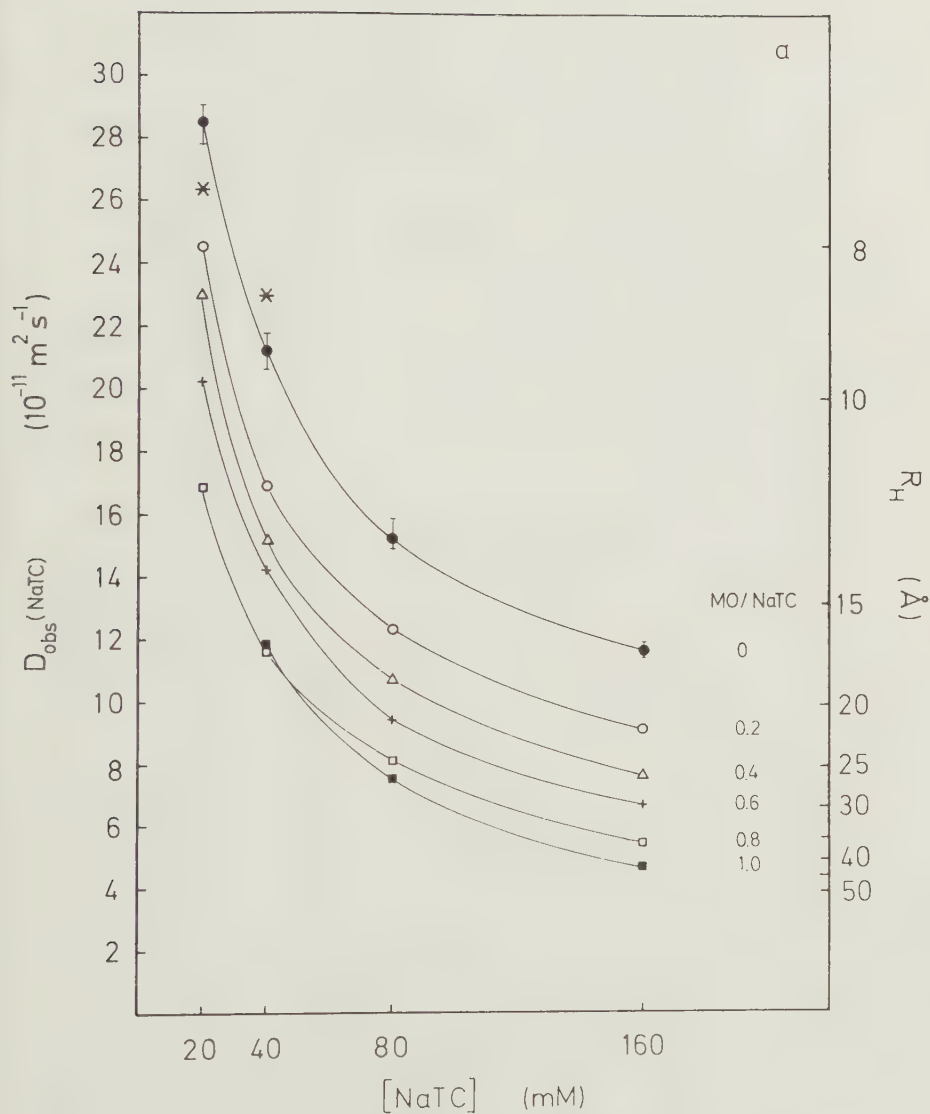


FIGURE 7a

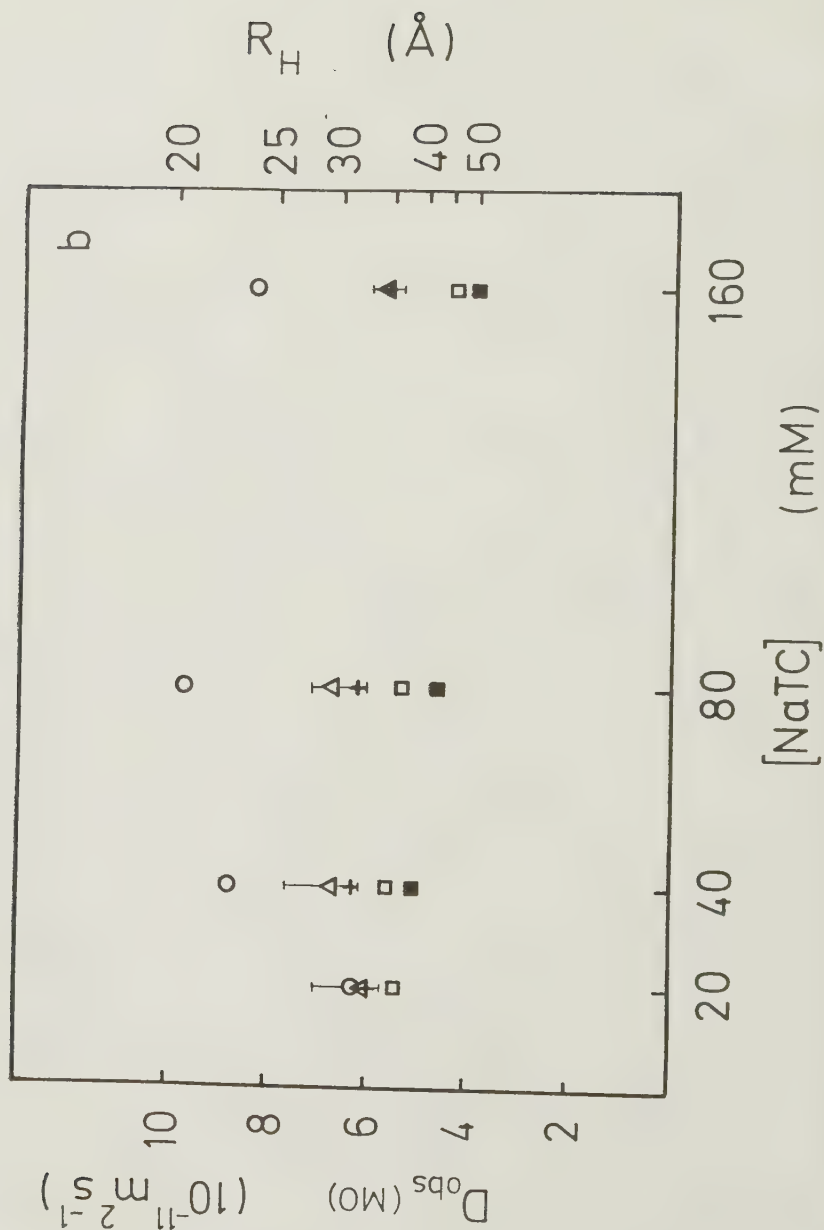


FIGURE 7b

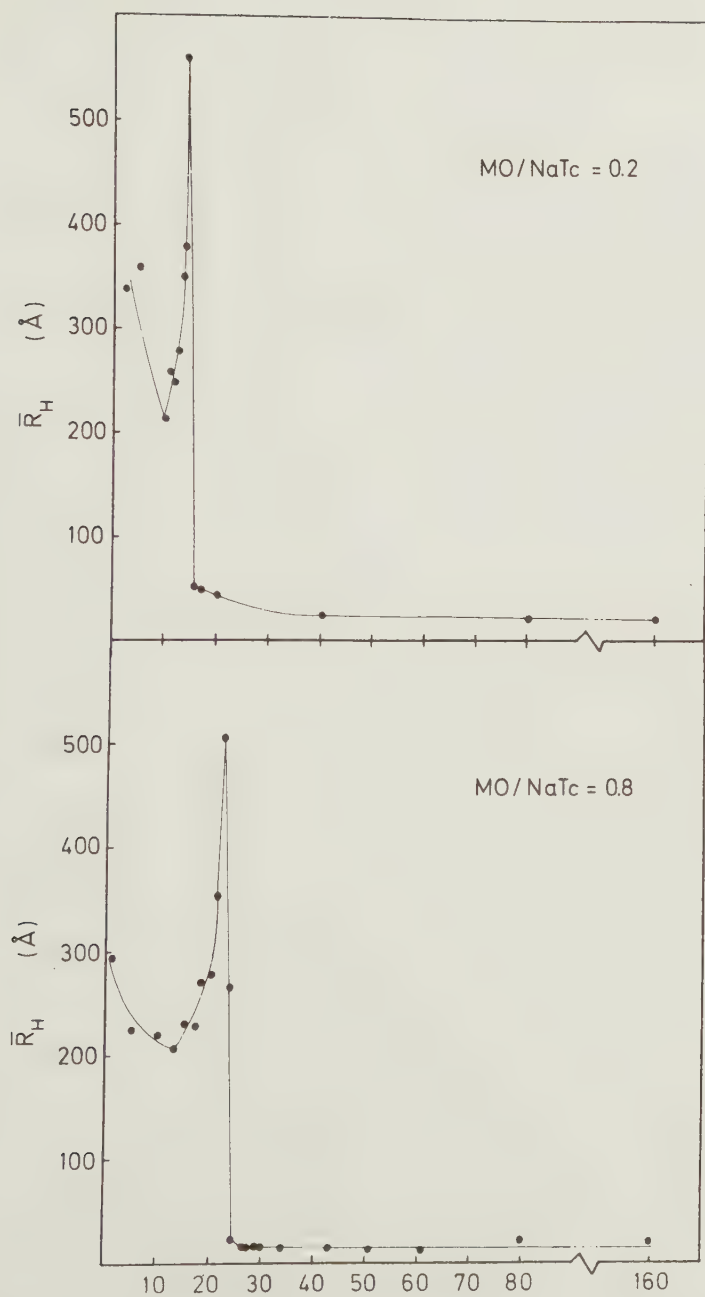


FIGURE 8

